Effects of guanidinoacetic acid and methionine on metabolism and performance in cattle

by

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B.S., University of Tehran, 2006
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AN ABSTRACT OF A DISSERTATION

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DOCTOR OF PHILOSOPHY

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Abstract

Efficient utilization of feed protein by cattle, because of its impact on economics and the environment, is a major concern for animal nutritionists and producers. Ruminants play an important role in food production (milk and meat) and are major sources of protein for human livelihoods. Two experiments were conducted to assess the value of guanidinoacetic acid (GAA) with or without methionine (Met) on nitrogen retention, methionine flux, and methylation reactions in cattle. As well, a study was conducted to investigate the effectiveness of 2 ruminally protected methionine sources for lactating dairy cows. Another study evaluated effects of abomasal infusions of casein on dairy cattle production.

The first experiment examined the effect of abomasal infusions of GAA (0, 10, 20, 30, or 40 g/d GAA) combined with either 0 or 12 g/d of methionine on creatine synthesis and plasma homocysteine concentrations in 6 ruminally cannulated Holstein heifers (520 kg initial body weight). This study demonstrated that plasma creatine was increased by GAA supplementation. Plasma homocysteine was not affected by GAA supplementation when heifers received 12 g/d methionine, but it was increased when 30 or 40 g/d of GAA was supplemented without methionine (GAA-linear × Met, $P = 0.003$). This experiment demonstrated that post-ruminal GAA supplementation increased creatine supply to cattle and spares arginine utilization.

In the second study, effects of GAA supplementation on nitrogen retention and methionine flux were evaluated in 7 ruminally cannulated Holstein steers (161 kg initial body weight) limit-fed a soybean hull-based diet. Treatments were provided abomasally and included 0 or 6 g/d of L- methionine and 0, 7.5, and 15 g/d of GAA. Steers received continuous abomasal infusions of an essential amino acid mixture devoid of methionine to ensure that no amino acid besides methionine limited animal performance, and energy was supplied by ruminal infusion of

volatile fatty acids and abomasal infusion of glucose. Whole body methionine flux was measured using continuous jugular infusions of 1-^{13}C-L- methionine and methyl-$^{2}H_3$-L- methionine. Nitrogen retention was elevated by methionine supplementation ($P < 0.01$). Supplementation with GAA tended to increase N retention when it was supplemented along with methionine, but not when it was supplemented without methionine. Supplementation of GAA increased plasma creatine concentrations GAA and creatine ($P < 0.001$). Loss of methionine through transsulfuration was increased by methionine supplementation, whereas synthesis of methionine from remethyation was decreased by methionine supplementation. No differences in transmethylation, transsulfuration, or remethylation reactions were observed in response to GAA supplementation. These results showed that administration of GAA, when methyl groups are not limiting, has the potential to improve lean tissue deposition and cattle growth.

In the third study, 21 Holstein dairy cows were utilized to study lactational responses of dairy cows to methionine provided from 2 ruminally protected sources of methionine activity. Treatments included: 1) control, 2 and 3) 7.5 and 15 g/d of a ruminally protected product of 2-hydoxy-4-methylthio-butyric acid (NTP-1401; Novus International, Inc., St. Charles, MO), and 4 and 5) 7.5 and 15 g/d of a ruminally protected DL-methionine product (Smartamine M; Adisseo, Alpharetta, GA). Milk protein percentage and milk protein yield increased linearly with supplementation, without differences between methionine sources or interactions between source and level. Plasma methionine concentrations were increased linearly by methionine supplementation, with the increase being greater for Smartamine M than for NTP-1401. Plasma D-methionine was increased only by Smartamine M. Plasma 2-hydoxy-4-methylthio-butyric acid was increased only by NTP-1401. These data demonstrated that supplementation with these

methionine sources can improve milk protein percentage and yield, and the 2 methionine sources did not differ in their effect on lactation performance or milk composition.

The final study was conducted to evaluate the effect of post-ruminal supplementation of casein on milk yield and composition and whole body protein deposition in dairy cattle. Two ruminally cannulated Holstein dairy cows (599 kg initial body weight) were used. Treatments included daily abomasal infusion of 0 or 400 g/d casein. Cows received 320 g/d glucose through continuous abomasal infusion to prevent energy from being limiting. This study demonstrated that abomasal casein infusion had no effect on milk yield and composition with the exception of milk protein percentage and milk urea nitrogen, which were increased ($P < 0.01$) when casein was supplemented. Nitrogen retention ($P = 0.03$) and urinary N excretion ($P < 0.0001$) were increased and fecal N excretion ($P < 0.0001$) was decreased by post-ruminal casein infusion. These results suggest that casein has potentially positive effect on stimulating protein deposition and can be used as an effective way to alter nitrogen utilization in lactating dairy cattle.

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Dedication

To my parents

Thank you for all of your unwavering love and support

Chapter 1 - Literature Review

Introduction

The major goals of any dairy producer are increasing milk production and production efficiency which can improve herd profitability. Efficient utilization of feed protein by cattle, because of its impact on economics and the environment, has become a major concern for animal nutritionists and producers. Ruminants play an important role in food production (such as milk and meat) and are major sources of protein for human livelihoods. The total feed conversion ratio to meat is much higher in ruminants (20:1) than nonruminants (3.8:1); therefore, ruminant animals are much less efficient in turning feed resources to meat (Wilkinson, 2011). The tissues and organs of ruminants require amino acids and energy for maintenance and productive functions; therefore, diet formulation strategies that meet protein and energy requirements of ruminants can enhance performance (e.g., growth, lactation, pregnancy, etc.) and efficiency of energy utilization.

Historically, ruminal requirements for protein were estimated by National Research Council (NRC) as dietary protein concentrations based on animal responses to gradual changes in dietary levels of protein (Tedeschi et al., 2015). Body weight gain consists of both fat and lean gain; thus, adequate supply of protein and energy, which support the synthesis and deposition of protein in tissues, has positive effects on live weight gain of cattle. In growing muscle, the rate of protein synthesis is more than protein breakdown, which leads to protein accretion (Owens et al., 1995; Weinert, 2009). Thus, the synthetic amino acids play critical roles not only because of increasing the availability of amino acids (nitrogen) for the animal, but also improving lean meat production (Beski et al., 2015). There are several nutritional approaches to optimize the postruminal supply of amino acids, and these include: 1) supplying a sufficient amount of the

dietary crude protein to provide the optimal amounts of rumen degradable protein (RDP) for increasing microbial protein synthesis and of rumen undegradable protein (RUP) which is resistant to ruminal degradation to improve quantities of amino acids reaching the small intestines; 2) supplementation with rumen-protected amino acids such as methionine; 3) providing a sufficient supply of energy to support microbial protein synthesis and improve the efficiency of feed nitrogen utilization. When the balance of nitrogen is zero (no demand for weight gain, losing body condition to supply the energy, and not turned into milk N), the unutilized nitrogen will be excreted in the feces (the undigested dietary protein fraction) and urine (absorbed by animal but not used for growth and milk and meat production). The conversion efficiencies for feed N into milk N rarely exceed 30%, which means more than 70% of N from the feed ends up in feces (30%) and urine (40%, mostly in the form of urea) (VandeHaar and St. Pierre, 2006). Lapierre and Lobley (2001) reported that the average of synthesized urea excreted in urine and recycled to the gastrointestinal tract were approximately 33% and 67%, respectively, and about 50%, 40%, and 10% of recycled urea are reabsorbed as AA, ammonia, and lost in feces. In addition, Chase et al. (2012) reported that in commercial dairy farms, the efficiency of converting feed nitrogen (N) intake to milk N is estimated between 20 and 35% with almost all the remaining nitrogen (between 65 to 80%) excreted in the manure. More precise feeding of protein and balancing of rations for providing an optimal balance of essential amino acids can improve the efficiency of utilization of N by reducing the CP content of the diet while ensuring the sufficient amounts of essential amino acids are provided (NRC, 2001). There is a linear relationship between energy intake and protein deposition, and the body protein deposition rate is not improved by dietary protein supply when energy intake is a limiting factor for protein deposition (Campbell et al., 1985; Schutz, 2011). For protein gain to be

efficient, the available essential amino acids should be sufficient to stimulate protein synthesis (Poppi and McLennan, 1995). Protein retention can be improved by increasing energy intake in preruminant calves, suggesting that the efficiency of AA utilization can increase with increases in energy supply (Gerrits et al., 1996). Physiological maturity is affected by genetics as well as nutritional and hormonal status and affects body tissue composition; therefore, the rates of protein accretion and fat synthesis may be affected by animalbody weight (Owens et al., 1995). Total protein conversion ratios were greater for lamb and beef than pork and poultry meat production (approximately 30 and 24 kg/kg versus 4.3 and 3.0 kg/kg total protein) (Wilkinson, 2011). The branched chain essential amino acids (BCAA), including leucine (Leu), isoleucine (Ile), and valine (Val), must be obtained through the diet because they cannot be synthesized by humans and in sufficient amounts in the ruminant. Loest et al. (2001), working with ruminally cannulated Holstein steers which were limit-fed soybean hull-based diets, found that BCAA deficiencies (Leu and Val) were able to limit protein deposition in steers fed diets deficient in ruminally undegradable protein based on soybean hull-based diets. Branched chain amino acids could increase protein synthesis (23%) and reduced protein degradation (-26%) (De Bandt and Cynober, 2006) and act also as a stimulator for insulin secretion (Holecek, 2010). Among BCAA, leucine is unique because of its specific function in mTOR signaling pathway activity, which can promote protein synthesis (Zhang et al., 2017). The dietary essential amino acids present in the body proteins and the total amino acids in muscle proteins are comprised approximately 35–40% and 14–18% of BCAA, respectively (Shimomura et al, 2006).

Protein and nitrogen metabolism in ruminants

Animal productivity, profitability, and N efficiency can be influenced by several factors such as dietary protein level and sources of protein and energy. Amino acids act as substrates for

protein synthesis and are vital for numerous biological and physiological functions in the body. Protein is present in high concentrations in muscle tissue; therefore, muscle is the major repository of the body's protein (Poortmans et al., 2012). Ruminants consume crude protein (CP) to supply nitrogen for microbial growth and amino acids (AA), which improve animal performance. The nitrogen requirements of the rumen microorganisms are provided via 1) dietary protein which is composed of rumen degradable protein (RDP; peptides, free amino acids, and ammonia) and rumen undegradable protein (RUP), 2) non-protein nitrogen (such as urea and nitrates), and 3) endogenous N (Lee and Beauchemin, 2014; Andrade-Montemayor et al., 2009; Bach et al., 2005). In general, the rumen degradable protein (RDP) is degraded in the rumen to amino acids and ammonia, which then is absorbed across the rumen wall into the blood stream to be detoxified by the liver as urea. Some urea can be recycled to the rumen via saliva or transfer from blood across the ruminal wall. Extra urea can be excreted in the urine. Some portion of RDP escapes degradation in the rumen and can pass to the small intestine to provide duodenal flow of AA and peptides (Hristov et al., 2004). Ruminally undegradable protein (RUP) is referred to as bypass protein that not broken down in the rumen but rather escapes rumen degradation and may be absorbed from the small intestine as AA or peptides and used for productive purposes (Merchen and Titgemeyer, 1992). Additionally, ruminally undegradable proteins could enhance the delivery of AA and flow of N to the small intestine (Titgemeyer et al., 1989), resulting in improving efficiency of N usage and growth rate (Goedeken et al., 1990). The amount of AA needed by a ruminant animal is determined according to expected levels of individual animal performance. It is important to note that ruminants can sufficiently synthesize all the AA via rumen microbes which convert low-quality dietary proteins into high-quality proteins (microbial proteins) more efficiently than monogastric animals (Varga and Kolver,

1997), although some amino acids cannot be synthesized in sufficient amounts by the ruminal microbes and may limit cattle performance; these are called limiting amino acids, and likely include methionine and lysine. Rumen microbes and dietary nitrogen that are not degraded in the rumen and pass to the intestine as significant sources of protein and amino acids for cattle. The activity of the microbial population of the rumen not only plays a crucial role to improve the efficiency of nitrogen metabolism, but also is important for the flux of amino acids into the small intestine via increasing the microbial protein synthesis which can improve ruminant productivity (NRC, 2001). Approximately 60 to 85% of amino acids entering the small intestine are supplied to ruminants from microbial protein flow to the small intestine, although it depends on the RUP and RDP content of the diet (Hackmann and Firkins, 2015; Block, 2006). In dairy cows, approximately 50 to 80% of the total protein is supplied through microbial proteins which are a good source of high quality amino acids (AA) and are also contain limiting amino acids for milk production, such as methionine and lysine (NRC, 2001). Also, the disappearance rates for total AA across the small intestine is estimated between 57% to 78% (averaged 70%) in lactating dairy cows (Calsamiglia et al., 2010). Proteins are degraded to volatile fatty acids (VFA), amino acids, carbon dioxide, and ammonia (NH_3), which is the major end product of protein-N and non-protein-N degradation in the rumen (Guliński et al., 2016; Andrade-Montemayor et al., 2009). The volatile fatty acids (propionic acid, acetic acid, butyric acid), which are absorbed across the ruminal wall into the blood stream, will be taken up by the liver (although acetate would not be taken up in high percentages by the liver) to be converted to other energy sources to carry out the thousands of functions in the body (Moran, 2005). The imbalance between the supply of energy (carbohydrate availability) and protein (N availability) in the rumen will decrease the efficiency of N utilization which leads to increases in both fecal and urinary N

excretion (mostly as urea) and inefficient N retention, which are potential sources of environmental pollution and feeding costs (Schroeder and Titgemeyer, 2008; Hristov et al., 2005). At least 1.2% N in the diet is required to allow normal rumen microorganism populations to have normal functioning. Ruminants have a remarkable ability for intra-ruminal N recycling, which will be discussed in further detail below; therefore, microflora can maintain the normal functions for several weeks when there is insufficient supply of dietary N for rumen micro-organisms (Pacheco and Waghorn, 2008).

Some studies have demonstrated that the level of dietary CP and energy can improve the rate of protein deposition until access to energy and amino acids do not restrict protein deposition (Valente et al., 2014). On the other hand, high dietary RDP, because it results in elevated ammonia levels in the rumen that are absorbed into the blood, can have a negative effect on cattle. Ammonia is almost completely removed from the blood with energy consumption (30 kJ ME/g of nitrogen) via hepatic elimination on the first pass through the liver; this process indicates that there is a positive correlation between energy and nitrogen metabolism (Pacheco and Waghorn, 2008). Overall, there are important factors which can have profound effects on rumen ammonia concentrations such as the quantity, source, and digestibility of protein that are ruminally available for microbes and energy sources such as fermentable carbohydrates.

Nitrogen efficiency is relatively low for milk protein synthesis and averages between 25% and 28% in North America and northern Europe according to dairy cow trials, despite the management strategies for maximizing genetic potential and diet strategies to increase feed efficiency via improvement of diet formulation (Huhtanen et al., 2014). A meta-analysis (Huhtanen and Hristov, 2009) reported that dietary CP, balancing ration for protein, and estimated MP are the major determinants of milk protein yield and efficiency of dietary N

transfer to milk protein in the dairy cow. In fact, inefficient utilization of protein by cows leads to N excretion to the environment and high feed costs. Especially in high-producing dairy cows need high-quality protein for optimum production. Therefore, because of increased demand for protein, the excess dietary protein can lead to overfeeding and elevated N excretion to the environment. Feeding a large amount of high quality protein when the considerable portion of nitrogen is not incorporated into microbial protein may increase the rumen ammonia pool and enhance urinary N excretion (Sannes et al., 2002). In a study by Chiavegato et al. (2015), Holstein steers fed rations containing 10% CP had less ammonia excretion than those fed a ration containing 13% CP, but no differences were observed for nitrous oxide and CH_4 emissions. Interestingly, there was no significant difference between treatments for starting and ending BW or DMI. James et al. (1999), working with Holstein heifers fed two levels of protein (9.6 or 11.0%), found that heifers fed lower protein diets had 29.6% less urinary urea N and 19.9% less total N excreted in the urine, resulting in reduced emissions of ammonia by 28%. Also, Broderick et al. (2008) fed rumen-protected methionine at 0, 5, 10, 15 g/d with diets containing 18.6%, 17.3%, 16.1%, or 14.8% CP and demonstrated improved N efficiency and reduced levels of milk urea N and urinary N excretion in multiparous Holstein cows fed the lowest CP diet containing the most rumen-protected methionine.

Protein degradation in the rumen, because of deamination, not only is the main cause of inefficient N retention, but also leads to increased nitrogen excretion by ruminants; thus, an assumption has been that the N source plays an important role in protein deposition status. Generally, there are concerns about low protein intakes, which may increase the risk for low dry matter intake (DMI), milk production, or/and milk protein yield, by cattle. However, the use of new technologies, such as ruminally protected amino acids, and nutritional manipulation, such as

feeding low protein diets with appropriate supplementation of essential amino acids (EAA) such as methionine, lysine, and histidine, not only may be the best way to improve animal N utilization efficiency but also may mitigate nitrogen excretion and ammonia emissions and the possible adverse consequences of low protein intake on cow performance (Giallongo et al., 2016; Giallongo et al., 2015). In ruminants, excess nitrogen can increase ruminal concentrations of ammonia and reduce the availability of AA and dietary AA utilization efficiency, because of being used to provide α-amino N for hepatic ammonia detoxification, which ultimately causes decreased production and overall performance (Lobley et al., 1995). In contrast, Awawdeh et al. (2006), McCuistion et al. (2004), and Hussein et al. (2016) reported that there is no negative association between excess ammonia and protein deposition when methionine (Met), leucine (Leu), histidine (His), or lysine were limiting for protein deposition by steers. Supplementation of a mix of essential and nonessential amino acids improved His utilization in cattle (McCuistion et al., 2004). Importantly, evidence suggests that differences in the efficiency of utilization of AA such as Met, Leu, and His may be because of inherent differences between AA which arises from differences in the rate of AA oxidation and/or amino acid metabolic process throughout the body (Batista et al., 2016). Calsamiglia et al. (2010) argued that the average efficiency of N utilization in ruminants was approximately 25%; however, there was considerable variation (15% to 40%) between experiments because of experimental conditions, such as dietary crude protein and metabolizable energy concentrations, and other factors.

The efficiency of feed crude protein utilization in dairy cows is greater than other ruminants, but excretion of manure nitrogen (N) is 2 to 3 times more than the amount of N excreted in milk (Broderick, 2007), which can increase milk production cost and lead to environmental pollution by releasing excess nitrogen to the environment. It is well established

that feeding diets with excessively high crude protein (CP) concentration to meet metabolizable protein (MP) and metabolizable amino acids requirements increases fecal and urinary nitrogen excretion (Muck, 1982; Erickson and Klopfenstein, 2001); therefore, the accurate determination of optimum protein and amino acid (AA) requirements and protein intake are critical to minimize nitrogen excretion and maximize efficiency of N (Huhtanen and Hristov, 2009; Lee et al., 2012).

Although the efficiency of N utilization in ruminants is lower than non-ruminants, there are several feeding strategies to minimize nitrogen losses, which are considered to induce several beneficial effects such as reducing environmental pollution and improving the efficiency of N utilization by ruminants. Some of these strategies include 1) feeding lower protein diets to prevent feeding an excess above the amount the cattle actually need, 2) optimizing the capture of nitrogen by rumen microbes, which results in improving microbial protein synthesis and efficiency of N, and 3) changing the quantity, type, and digestibility of carbohydrate and protein in the diet to improve energy and N availability for microbial synthesis and escape protein from ruminal degradation to enter the small intestine (Reynolds and Kristensen, 2008).

Nitrogen and environment

As the human population grows from its current level, significant investments are needed to improve agricultural production. United Nations (2017) reported that the global population of 7.6 billion in 2017 is expected to reach 9.8 billion in 2050, approximately 27 percent higher than in 2017. With this growth, the global food demand (such as meat, eggs, dairy products, vegetables, and fruits) will be higher than today and food production needs are expected to increase by 70 percent as a result of increasing human consumption, changes in diets, rural-to-urban migration, and climate change. Animal agriculture and its environmental impact have been a major concern worldwide. The increase in agricultural productivity could have an impact on the environment,

such as nitrogen compounds emitted into the environment having negative impacts on the ecosystem and human health. Therefore, improving nitrogen use efficiency can be beneficial to the environment through reducing animal excretion of manure N and also can improve productivity of livestock such as yields of milk and meat. In the United States and Canada, agriculture is the major source of ammonia emissions, almost three-fifths of NH_3 emissions into the environment (Bittman and Mikkelsen, 2009). Livestock farming is an important contributor of ammonia to the environment. Cattle farming, especially dairy husbandry, has been regarded as the largest source of ammonia emissions (Meisinger and Jokelo, 2000). The rate of volatilization of urine N is usually greater than that of fecal N because urine N can quickly convert into ammonia, which can be harmful to the environment (Panetta at al., 2006). Less than 50% of the total nitrogen consumed by animals is excreted and lost to the atmosphere as NH_3 (Carter et al., 2012). Ammonia is a colorless gas that is lighter than air and has sharp odor. Approximately 64 to 86% of global anthropogenic ammonia emissions originate in livestock, while cattle CAFO (concentrated animal feeding operations) are responsible for 43 to 48% of ammonia production (Waldrip et al., 2015b). Liu et al. (2017) also reported that livestock production accounts for more than 80% of ammonia emissions in the U.S., while more than 50% of ammonia comes from beef and dairy cattle. The manure nitrogen can be characterized into inorganic (ammonia, nitrites, and nitrates) and organic (urea and undigested protein) forms; however, the organic N forms are the most predominant constituent of animal manure, while a negligible amount of inorganic N is found in animal manure (USEPA, 2004). The largest fraction of nitrogen in urine is in the form of urea, which accounts for about 24 to 71% of total N excretion by finishing cattle (Waldrip et al., 2015b). In ruminants, conversion of dietary N to animal products such as milk and meat is often inefficient; for example, the rate of deposition of

consumed N in feedlot cattle body tissues was only 10% to 20% of the N, whereas approximately 80% to 90% percent of consumed N was excreted in manure, with approximately 60 to 80% in urine and 20 to 40% in feces (Chiavegato et al., 2015; Dong et al., 2014; Jonker et al., 2002). There are several possible factors that can cause the variability in emissions, including nutrition, dietary composition, manure storage and handling systems, and environmental conditions (Chiavegato et al., 2015). Observational evidence indicated that a 50% improvement in nutrient utilization efficiency via better herd management leads to a reduction in nitrogen excretion of up to 40% into the water or environment (Jonker et al., 2002). Nitrogen volatilization as NH_3 from manure causes not only reduction in fertilizer value of manure but also atmospheric pollution, with the amount of ammonia lost by volatilization estimated at between 5% to 80% of the manure N (Liu et al., 2017).

It is obvious that the majority of climate change is because of greenhouse gases (GHG), which include carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and water vapor (H_2O); 35–40% of CH_4 and 65% of N_2O are produced from enteric fermentation and farm animal manure, and these account for more global warming than any other gases (Oonincx et al., 2010). As the concentration of urea in urine is greater than feces (Liu et al., 2017), the reduction of the excretion of urinary N can reduce environmental pollution because of decreased ammonia emissions from livestock manure (Kebreab et al., 2010). Immediately after excretion, because of contact between feces and urine after excretion, urinary urea is hydrolyzed to NH_3 through catalytic activity of urease enzyme, which is predominately found in feces (Todd et al., 2013). Variations in urea concentration, soil properties, pH, and temperature can affect urease activity. For example, the urease activity increased with increasing temperature from 10 to 40°C. Although urease activity is reduced within the pH range of 6.8 to 7.6 (Hristov et al., 2011a), the

rate of ammonia volatilization was higher in the range of 7.5 to 8.5 (USEPA, 2004). Excess nitrogen can cause damage to the soil via nitrification, the oxidation process by which ammonia is oxidized to nitrite and nitrate by nitrifying bacteria in the soil. The reduction of nitrate to N_2O and N_2 and also the utilization of NH_3 and NH_4^+ by plants leads to excretion of H^+ from roots (soil acidification) not only can be major mechanisms for nitrogen loss but also play an important role in drastic changes in quality of water (water pollution) (Waldrip et al. 2016; Oonincx et al., 2010; Bittman and Mikkelsen, 2009). When nitrogen enters a body of water, it can stimulate excessive aquatic plant and algae growth which reduces the level of dissolved oxygen for fish and other aquatic life and eventually can cause loss of marine biodiversity, ecosystem and water quality disruption, and fish kills (Waldrip et al., 2015; Ghaly and Ramakrishnan, 2015). Overall, the elimination of excess nitrogen through animal manure (urine and feces) is costly in terms of environmental pollution.

Urea Cycle

The ability of ruminants to recycle considerable quantities of urea to the rumen increases the efficiency of N use, microbial protein synthesis, and body protein deposition (Valkeners et al., 2008). Recycling of urea to the rumen can be a useful strategy to improve the efficiency of N utilization in ruminants consuming low-N diets to support microbial protein synthesis (Pacheco and Waghorn, 2008). Ruminants fed low-protein diets have greater proportions of urea recycled than animals fed diets containing adequate amounts of protein (Marini et al., 2004). As already said, deamination of amino acids by ruminal bacteria lead to ammonia production, which is correlated with dietary N intake. Also, the urea transport rates across the rumen epithelium are dependent on many factors including luminal ammonia and pH and dietary N intake. The urea net transfer across the rumen wall is correlated with plasma urea-N (PUN); luminal urea

concentrations are relatively low because of ruminal urease activity, which is a major modulator of urea transfer (Muscher et al., 2010). Also, recycling blood urea nitrogen (BUN) to the gastrointestinal tract (GIT), because of positive association between the transport of nitrogen across the rumen epithelium and BUN concentration, can have an important role in maintaining ruminant animals in positive N balance (Walpole et al., 2015). Walpole et al. (2015) noted that feeding diets containing higher levels of ruminally fermentable carbohydrate (Huntington and Archibeque, 1999) or more extensive processing of grain (Delgado-Elorduy et al., 2002; Theurer et al., 2002) increased the entry of urea-N to the gut which increases ammonia-N availability to ruminal microbes and, consequently, reduces BUN because of reduced absorption of ammonia into the blood stream. There are varying quantities of recycled nitrogen from 25% of nitrogen ingested to 90% of urea turnover (Abdoun et al., 2010), while the averages of recycled nitrogen were 93%, 88%, and 161% of nitrogen ingested in growing cattle, lactating dairy cows, and young mature sheep, respectively (Lapierre and Lobley, 2001). Increased ruminal ammonia concentration reduces urease activity and thereby can inhibit urea-N entry when ruminal ammonia concentrations are elevated. This reduces the urea concentration gradient and consequently transfer across ruminal epithelia. Therefore, ruminal ammonia concentration has direct effects on the urea transporters of the ruminal epithelium. Increases in ruminal ammonia lead to decreased activity of the urea transporters (Muscher et al., 2010).

Diet composition affects recycling of urea to the gut and excretion of urea in urine. When steers received a low-protein diet, 2.3% of urea production was excreted in urine and 97.7% of the urea was recycled to the gut (Wickersham et al., 2008). It is possible that high-protein diets may have an inhibitory effect on urea-N recycling to the rumen and consequently have a negative impact on urea transport rates (Lu et al., 2014). Generally, ammonia in the rumen

disappears via three ways: 1) incorporation into microbial proteins, 2) absorption across the rumen wall (between 35–65%), and 3) rumen fluid outflow into the omasum (approximately 10%) (Abdoun et al., 2006). The portal vein carries absorbed ammonia directly to the liver where about 70-95% of ammonia is converted to urea or glutamine (Gln). The availability of energy can impact urea N recycling via improving the efficiency of capture of recycled urea N by the microbial population in the rumen (Parker et al., 1995).

Urea transporter (UT) proteins such as urea transporter-B (UT-B), which play an important role in transferring urea to the rumen from blood down a concentration gradient, have a critical role in the transfer of urea through the rumen epithelium. Dietary intake also has a regulatory role on both location and abundance of ruminal UT-B protein within the ruminal papillae (Walpole et al. 2015). VFA or CO_2 increased the ruminal urea transport rates via UT-B when rumen pH was 6.6 or 6.2 compared to pH 7.4 (Abdoun et al., 2010). However, inhibition of UT-B did not completely block urea permeability, indicating that multiple mechanisms may be responsible for the transport of urea across the rumen; for example, the aquaporins (AQP) belong to a family of membrane proteins that function as water channels, but a few of these (AQP-3, AQP-7, and AQP-10) are also permeable to urea (Litman et al., 2009; Barbara, 2010).

VFA are important, as an energy source, in stimulating NH_3 absorption across the rumen epithelium. Intraruminal VFA levels can increase flux across the rumen wall which was likely due to changes in ruminal epithelial permeability or/and increase rumen epithelial blood flow, resulting in increased NH_3 absorption. Indeed, observational evidence by other authors indicates the positive effects of butyrate on gastrointestinal tract, including decreased glucose and glutamine oxidation and increased tight junction gene expression and blood flow (Foote and Freetly, 2016; Abdoun et al., 2006; Baldwin et al., 2004). Furthermore, Remond et al. (1993)

reported when butyrate was injected into the rumen, it increased ruminal blood flow by 60% and NH_3 absorption from the rumen by 40%, but decreased net-uptake rate of urea by 30%. It has been speculated that there is a negative association between NH_3 absorption and net transfer of urea (Remond et al., 1993). In addition, urea can be recycled to the rumen through saliva, and forage concentration has an important impact on the salivary secretion rate (Lapierre and Lobley, 2001). Huntington (1989) showed that the amount of ruminal urea that was derived from saliva was greater in alfalfa hay-fed cows (69%) than in cows fed a concentrate diet (23%), which may relate to reducing dietary forage content.

Overview of Methionine

History of Development

In 1847, the first detection of sulfur-containing amino acids was described by Fleitmann and the reduction in heat stability and the liberation of H_2S and NH_3 were observed for sulfur-containing proteins in strong alkaline solutions (Willke, 2014). Nearly 54 years later, two sulfur-containing AA were discovered; one was cysteine (Osborne, 1902), and the other, first isolated from casein, was identified as methionine by Mueller in 1923. Dietary methionine can be degraded by rumen microbes and the profile of absorbed Met is often less than ideal in the ruminant. Methionine is considered one of the most limiting amino acids for protein synthesis for dairy cow production (Varga, 2010; Schwab et al., 2003); therefore, ruminally undegradable Met is an advantageous way to improve dairy cow performance by providing higher concentrations at the site of absorption and reducing loss and waste of dietary N. Because of this, in the 1960's and early 1970's, attempts were made to produce ruminally protected methionine (Schwab, 1995).

Methionine in the ruminant

Amino acid nutrition of dairy cows has received considerable attention in enhancing performance over the last decade, resulting in ration formulation to optimize the efficiency of protein utilization and prevent overfeeding of proteins. Ruminants are fed diets designed to meet their nutrient and crude protein (CP) requirements, but quantities of some amino acids reaching the small intestine of ruminants are inadequate to meet the cow's needs, and this can limit protein accretion and overall animal performance. Supplementation of amino acids may be needed to meet requirements for essential amino acids. Supplementation with RUP may be an effective way for providing limiting amino acids to satisfy the animal's requirements. Methionine and lysine are considered as the first and second limiting amino acids for protein synthesis and optimized beef and dairy cow production (Noemí et al., 2017; Varga, 2010; Schwab et al., 2003; Titgemeyer et al., 1989; Richardson and Hatfield, 1978). The AA profiles of RUP sources vary from one feedstuff to another and the types of ingredients used in the diets have important roles in determining limiting amino acids for ruminants (Merchen and Titgemeyer, 1992). For example, meat and bone meal and corn gluten meal (CGM) both are important sources of RUP, but meat and bone meal is deficient in methionine, whereas corn gluten meal is low in lysine (Klemesrud et al., 2000 b; Titgemeyer et al., 1989). Blood meal (BM) also is a good source of RUP, but it is deficient in sulfur amino acids (SAA) (Klemesrud et al., 2000a). Abe et al. (1997) showed that lysine was the first limiting amino acid for calves roughly less than 3 mo of age which were fed corn- or corn gluten meal-based diets. Also, it has been reported that increased postruminal supply of limiting AA such as methionine can improve muscle, leather, and wool growth as well as N retention by enhancing the efficiency of nitrogen utilization in sheep and cattle (Archibeque et al., 2002; Schelling et al., 1973). Merchen and

Titgemeyer (1992) reported that a mixture of essential amino acids, which provides an adequate proportion of all the essential amino acids, can improve nitrogen retention. The improvement in nitrogen retention was greater when limiting amino acids, such as methionine, were utilized in combination with other essential amino acids compared with methionine alone (Awawdeh et al., 2006). Average daily gain (ADG) and protein efficiency increased when steers fed meat and bone meal (MBM) were supplemented with rumen-protected methionine (RPM) (Klemesrud et al., 2000a,b). Additionally, Klemesrud et al. (2000b) concluded that the ADG of growing steers deficient in metabolizable protein can be improved by dietary supplementation of rumen-protected amino acids; thus, accurate estimates of the ideal amino acid profile and limiting amino acid requirements are useful to achieve improvement in rates of gain and efficiency of protein utilization.

Lactating dairy cows have specific requirements for essential amino acids (EAA) to improve lactation performance and efficiency of N use (Brake et al., 2013; Ordway et al., 2009). The nutritive profile of essential amino acids, particularly lysine and methionine, are considered to determine the MP value of feed ingredients for dairy cows (Das et al., 2014). Methionine is considered one of the most limiting amino acids for optimized dairy cow production (Varga, 2010; Schwab et al., 2003) and evidence suggests that dairy cattle during late gestation have the highest requirements for methionine (Bach et al., 2000). Feeding unprotected methionine is typically not considered an acceptable practice for ruminants due to the high rates of degradation within the rumen. Ruminally undegradable Met is therefore an advantageous way to improve dairy cow performance by providing higher concentrations at the site of absorption and reducing loss and waste of dietary N.

Increasing Met supply through postruminal infusions of methionine was effective at increasing protein synthesis in the mammary gland of lactating dairy cows (Guinard and Rulquin, 1995; Pisulewski et al., 1996; Socha et al., 2008). Supplemental sources of Met to increase postruminal supplies of Met are rumen-protected methionine (RPM) and Met hydroxy analogs such as 2-hydroxy-4-methylthio butyric acid (HMTBa). Ardalan et al. (2010) reported that supplementation of RPM could improve reproductive performance and health status of dairy cows. A meta-analysis (Zanton et al., 2014) of supplemental dietary Met sources such as RPM and HMTBa, or postruminal infusion of Met in dairy cows indicated Met increased milk protein content and yield across 64 studies in the literature. A meta-analysis (Patton, 2010) based on 35 studies feeding RPM to dairy cows also indicated that adding Met to the diet increased milk protein content and yield, and milk yield was slightly increased. As a consequence of beneficial effects of Met supplementation, such as increased milk protein content and yield, technologies have garnered much attention in recent years to prevent Met degradation in the rumen by ruminal bacteria (Brake et al., 2013). There are several RPM products that are available commercially around the world to improve the performance efficiency of lactating cows.

Methionine Hydroxy Analog

The protection of Met allows it to be delivered into the small intestine without degradation in the rumen and to be available for intestinal absorption. Researchers have conducted a number of significant efforts in order to meet the methionine requirements of cattle; hence supplementation of rumen-protected forms of methionine can be a beneficial approach to support the requirements of cows, especially dairy cows. There are some commercial Met products such as RPM, DL-Met, HMTBa, or the isopropyl ester of HMTBa (HMBi), which Met derived from these is used to meet the needs for cattle performance. Rumen protected Met and

hydroxy analogs of methionine, such DL-2-hydroxy-4-methylthiobutanoate (HMTBa) and isopropyl ester of HMTBa (HMBi), can supply the Met requirements for dairy cows to improve lactational performance (Zhang et al., 2016; Vazquez-Anon et al., 2001). Methionine hydroxy-analog, which is a synthetic methionine-related compound and not a true amino acid, can be metabolized to methionine by cattle. The conversation of hydroxy-analog to L-Met can take place in a range of tissues, but Met synthesis rate was greatest in liver and kidney (Lobley et al., 2006). The isopropanol group of HMBi reduces its rumen degradability (St-Pierre and Sylvester, 2005), therefore the availability of methionine has been estimated to be approximately 50%-60% (Breves et al., 2010; Noftsger et al., 2005). Generally, the analog HMTBa was more resistant than L-Met to microbial degradation in the rumen (Lapierre et al., 2011). Also, the isopropyl group makes HMBi more lipophilic, such that a significant portion is absorbed across the rumen wall (Graulet et al., 2004). In addition, HMBi must be hydrolyzed to HMTBa and isopropanol before the conversion into L-Met. The ruminal degradation rate of HMTBa are reported to vary from 99% (Jones et al., 1988) to 21 to 50% (Koenig et al., 1999; Vazquez-Anon et al., 2001). In ruminants, it is possible that the metabolism sites of HMTBa may be ruminal and omasal epithelia, liver and kidney (Lobley et al., 2006). Further, HMTBa and HMBi after passage across the intestinal wall can be converted into 2-keto-4 (methylthio) butanoic acid by two separate enzymes: D-2-hydroxy acid dehydrogenase (D-HADH), found in many tissues, and L-2-hydroxy acid oxidase (L-HAOX), found mainly in liver and kidney, and then followed by transamination to L-Met for effective utilization (Fang et al., 2010). The metabolism of D-HMTBA can occur effectively in ruminant tissue; although, the higher activity of D-HADH (by 45% to 75%) than L-HAOX was observed in ovine omasum, rumen, and kidney (Zhang et al., 2015a). In an experiment conducted by Lapierre et al. (2011), ^{13}C-labeled HMTBa was infused intravenously

to four multicatheterized cows, and the results indicated that HMTBa increased whole-body Met availability and 15% of the milk protein Met and 3.8 mmol/h of Met flow through plasma were also provided through HMTBa. This demonstrates that HMTBa can act as the source of Met for cattle. In addition, the administration of HMBi has enhancing effects on milk production, milk protein percentage, and production of protein, milk fat percentage, lactose yield, N efficiency, and ruminal microbial activity (Chen et al., 2011; St-Pierre and Sylvester, 2005). Although, Fang et al. (2010) reported that the capacity of the intestine for oxidative activity to convert D-Met and D-HMTBa to L-Met is higher than that of L-HMTBa to L-Met in broilers which can be attributed to the greater activity of D-HADH than L-HAOX in the intestine. Metabolic differences between various methionine sources may be due to differential absorption, transport, and metabolism characteristics of the methionine sources (Zhang et al., 2015a).

Bioavailability of D-methionine relative to L-methionine

Amino acids consist of stereo isomers referred to as D- or L-isomers, and all AA, except for glycine, can occur in these two isomeric forms (Willke, 2014). A racemic mixture is a 50:50 mixture of D- and L-isomers (Nguyen et al., 2006). DL-Methionine refers to the racemic mixture (Dilger and Baker, 2007). The utilization efficiency of D- and L-isomers vary among different species; for example, chicks can utilize D-isomers of branched chain amino acids (leucine, isoleucine, and valine) more efficiently than rats, whereas both rats and chicks efficiently utilize the D-isomer of phenylalanine, tyrosine, and methionine (Baker, 1986). Generally, D- and L-Met can be used effectively by cattle to improve productive performance, although, for protein synthesis, only the L-isomer of Met is involved; therefore, D-Met must be converted to L-Met prior to protein synthesis (Lapierre et al., 2012; Campbell et al., 1996). D-Met undergoes oxidative deamination via D-amino acid oxidase, which removes the amine group, producing α-

keto-γ-methiolbutyric acid. Following that, a transamination reaction is required to generate L-Met through transferring an amine group by transaminases to α-keto-γ-methiolbutyric acid, which is an intermediate metabolite (Hasegawa et al., 2005). The research by Shen et al. (2015), who evaluated effects of feeding L-Met and DL-Met on 1-d-old Ross chicken's small intestine, demonstrated that L-Met had more beneficial effects on the development of the gut than DL-Met. In addition, D-amino acid oxidase was found only in the kidney and liver; therefore, the utilization of D-Met by the intestinal cells depends on the conversion of D-Met to L-Met in the liver and kidney. In young animals, L-Met can also be the only form usable by animals because of the very low level of expression of D-amino acid oxidase (Shen et al., 2015). Also, supplementation of D-Met leads to higher plasma Met concentrations than does supplementation of L-Met, because D-Met is not converted rapidly to L-Met (Campbell et al., 1996). In cattle, removal rates for L-Met were estimated to be 6-fold faster than those for D-Met (Lapierre et al., 2012). The rate of transformation of the D-Met to L-Met influences bioavailability of D-Met (Lapierre et al., 2012). Hasegawa et al. (2005) demonstrated that more than 90% of administered D-Met was converted into L-Met in rats. In addition, Kong et al. (2016) showed that bioequivalence of D- to L-Met based on urinary N output and N retention in pigs were 87.6% and 89.6%, respectively, following administration of methionine (0.4 or 0.8 g L-Met/kg and 0.4 or 0.8 g D-Met/kg) with a corn starch basal diet (just deficient in Met). Shen et al. (2015) working with 1-d-old chickens demonstrated that both L-Met and DL-Met had favorable effects on the development of the villus because of an increase in the concentrations of glutathione and total antioxidant capacity. Also, the comparison between L-Met and DL-Met showed that L-Met was more effective for redox status and intestinal development in the young chick (Shen et al., 2015). Fanatico et al. (2007), working with male birds of three genotypes with different growth

rates (slow, medium, and fast) and different dietary Met levels, illustrated that level of MET had a numerical effect on weight gain and feed efficiency. Higher breast yields were observed for diets containing high levels of methionine. Shen et al. (2014) conducted two experiments to compare the effects of dietary L-Met and DL-Met on growth performance and gut health in nursery pigs. That work demonstrated that morphology of the gastrointestinal tract and average daily gain of nursery pigs were improved and plasma urea nitrogen (PUN) reduced in pigs fed a diet supplemented with L-Met compared with DL-Met, and this may be associated with the biologically functional form of L-Met being utilized directly by cell.

Structure and Function

The side chain (R group) of amino acids affects their structure and function. Methionine has a side chain containing a methyl group for metabolism attached to a sulfur atom; therefore, it exhibits hydrophobic and electrophilic characteristics because of a terminal methyl group and sulfur (Brosnan and Brosnan, 2006). The highly hydrophobic character of methionine, because of a terminal methyl group, makes methionine one of the most hydrophobic amino acids. More than two-thirds of the methionine residues in globular proteins are located on the interior of protein (hydrophobic), whereas approximately one-third can be found on the outside of a protein (hydrophilic) (Brosnan and Brosnan, 2006). In mammals, methionine has been identified as an essential amino acid for growth and development, and methionine is the first limiting amino acid for growing cattle and lactating dairy cows (Richardson and Hatfield, 1978; Schwab et al., 1976).

In addition to its role in protein synthesis, methionine performs a variety of biological functions in the body such as methylation reactions (where it acts as methyl donor group) such as synthesis of phosphatidylcholine, precursor for polyamine synthesis, a sulfur donor, antioxidants (such as glutathione and taurine), and precursor for sulfur amino acids such as cysteine (Chen et

al., 2014; Mato et al. 2013; Ardalan et al., 2011, Loest et al., 2002). Methionine can come from both diet and remethylation of homocysteine (Robinson et al., 2016). Methionine is metabolized through 3 major pathways: transmethylation, transsulfuration, and aminopropylation. The transamination pathway can also be considered as a catabolic pathway for methionine, and the activity of the transamination pathway depends on the methionine concentration. It is a minor pathway in normal conditions, but it becomes more important under condition of high methionine levels where it acts as a mechanism for degradation and clearance of methionine (Brosnan and Brosnan, 2006). For this reason, the discussion is limited to the 3 major pathways.

Methionine Metabolism

Methionine, choline, and betaine are three major dietary sources for methyl groups in the body (Kettunen et al., 2001). Choline acts as a precursor to betaine by the action of choline dehydrogenase (choline oxidase). Betaine provides a methyl group for the formation of Met from homocysteine. Thus, choline not only can spare methionine but also can act as a methyl donor in the body. The important roles of betaine and choline metabolism for the functions of cell membranes, hepatic lipid transport, methylation of homocysteine to form methionine, and cholinergic neurotransmission have been highlighted (Zeisel, 2006). There are important interrelations among the choline, methionine, and betaine pathways.

The major pathways for methionine metabolism in mammals are transmethylation, remethylation, and transsulfuration. Methionine adenosyltransferase (MAT) is the most important enzyme for methionine catabolism. In mammalian tissues, there are three MAT isoenzymes: MAT I and MATIII in the liver and MAT II in other tissues (Corrales et al., 2002). The liver is the major site for synthesis and metabolism of SAM (S-adenosyl-L-methionine) and methionine metabolism. More than 85% of methylation reactions occur in the liver, and high

methionine concentrations may contribute to increased production of SAM (Lu and Mato, 2012). Supporting this proposal is the observation that the MAT III activity appears to be increased by high methionine concentrations in the liver (Lu and Mato, 2012; Finkelstein, 1990).

Methionine is activated by MAT, using L-methionine and ATP as co-substrates to form S-adenosyl-L-methionine (also abbreviated as SAM, SAMe, or AdoMet), which is the primary methyl group donor and acts as a key biological molecule. Therefore, methionine has a role as a methyl donor for many transmethylation reactions such as choline synthesis and the formation of creatine from guanidinoacetic acid. S-adenosyl-L-methionine inhibits MAT-I and MATII by feedback inhibition and activates MAT-III. Thus, SAM plays a key role in the regulation of MAT I/II/III activities and methionine metabolism (Corrales et al., 2002; Finkelstein, 1990). Following methyl group donation, S-adenosyl-L-homocysteine (abbreviated as SAH or AdoHcy), resulting from the transmethylation reaction by the enzyme glycine N-methyltransferase (GNMT) or any of hundreds of other transmethylation reactions, is generated from SAM and hydrolyzed to homocysteine (Hcy) in a reversible reaction (Lu and Mato, 2012). The SAM:SAH ratio is an important parameter because it indicates the rate of flow of methyl groups within cells and can be used as a measure of cellular methylation potential (Ganguly and Alam, 2015). S-Adenosylhomocysteine has inhibition properties for methyltransferases (Brosnan and Brosnan, 2006). Methionine can be regenerated from homocysteine in the liver and kidney by betaine-homocysteine methyltransferase (BHMT) via a folate/B_{12}-independent pathway or in all tissues by methionine synthase via a folate/B_{12}-dependent pathway (Feng et al., 2011; Miller, 2003; Lieber and Packer, 2002). Consequently, the methionine requirement must consider not only the needs for protein synthesis but also those for transmethylation and transsulfuration reactions in the body. The sufficient supply of methionine is dependent on the availability of

methionine, which is provided through the diet or by homocysteine remethylation using folate or betaine.

The interrelationship between methionine, betaine, folate, vitamin B_{12}, and homocysteine metabolism

There are interrelations among metabolic pathways of methionine, choline, and betaine, which are also dependent on vitamin B_{12} and folic acid as cofactors. Methionine, choline, and betaine play key roles as methyl donors in transmethylation reactions in the body. Homocysteine is synthesized as a byproduct of transmethylation reactions involving SAM; therefore, an improved understanding of the mechanisms of methionine metabolism should be valuable to develop means of improving feed efficiency of a herd by allowing more accurate determination of optimum protein and amino acid (AA) requirements.

Folate and Vitamin B_{12}

Folate (vitamin B_9), which is naturally present in a wide variety of foods such as fruits and vegetables, is a water-soluble B-vitamin that plays an important role as a vitamin in crucial cell processes and acts as donor and acceptor of one-carbon units in folate-dependent one-carbon metabolism (Choi and Mason, 2000). Folic acid is the synthetic and stable form of folate which is used as a dietary supplement and does not occur in nature (Winkels et al., 2007; Ohrvik and Witthoft, 2011). Food folates, which exist in polyglutamate forms, must be hydrolyzed to monoglutamates prior to absorption in the small intestine, where the proximal small intestine is the main absorptive site. The bioavailability of the polyglutamate form has been estimated to be approximately 60–80% compared with the monoglutamate form (Melse-Boonstra et al., 2002; Visentin et al., 2014). Absorbed monoglutamates are transferred to the portal vein to be taken up by the liver (Said, 2011; Visentin et al., 2014), which contains around half the body's folate

(Gregory et al., 1998). Folate plays an essential role in several metabolic pathways such as purine-pyrimidine and DNA synthesis, and remethylation pathway for the regeneration of methionine from homocysteine.

Vitamin B_{12} (cyanocobalamin) is a water-soluble vitamin. Neither plants nor animals are capable of producing B_{12}, and it is made only by bacteria and archaea which contain the enzymes necessary for cyanocobalamin synthesis (Mahmood, 2014). Previous research has shown that vitamin B_{12} is crucial for proper folate functioning. Vitamin B_{12} plays an important role as a coenzyme for methionine synthase (MS) in transferring the methyl group from 5-methyltetrahydrofolic acid (MTHF) to homocysteine (Gröber et al., 2013). Folate and/or vitamin B_{12} deficiency or disruption of folate and B_{12} metabolism can diminish methionine-homocysteine biosynthesis (Malouf et al., 2003; de Koning et al., 2016) and lead to decreased methylation, decreased concentrations of SAM, and increased homocysteine concentrations (Stover and Garza, 2002). Also, vitamin B_6, because of its function as a cofactor, is important for the conversation of homocysteine to cystathionine through the transsulfuration pathway (Welch and Loscalzo, 1998). Additionally, vitamin B_6 is a cofactor necessary for the formation of 5,10 methyl-tetrahydrofolate (5,10- MTHF) from tetrahydrofolate (THF) and is therefore involved in folate metabolism (Welch and Loscalzo, 1998). Other research also reported that administration of folate, B_6, and B_{12} have a striking effect on reducing elevated homocysteine levels in patients with hyperhomocysteinemia (Brattstrom et al., 1988; Welch and Loscalzo, 1998; van Oort et al., 2003). It is obvious that folate, B_6, and B_{12} play important roles not only in the regulation of homocysteine metabolism and in controlling blood concentrations of homocysteine, but also in remethylation of Hcy to Met (McCully, 2007). Vitamin B_{12} and B_6 deficiencies, because of their role as cofactors for folate-dependent enzymes, can cause impairment of folate metabolism

(Stover and Garza, 2002). In ruminants, ruminal microorganisms may sufficiently synthesize B-vitamins (NRC, 2001). Although rumen microorganisms normally synthesize B-vitamins, during early lactation in dairy cows there is a large demand for methyl groups; thus, administration of B-vitamins (such as folate and B_{12}), if sources of methyl groups are adequately supplied, may be beneficial to improve remethylation of homocysteine to methionine (Girard et al., 2005). Previous studies have shown that folic acid and vitamin B_{12} supplemented alone or in combination could improve milk production and milk component yield in dairy cows (Girard et al., 1995, Girard and Matte, 1998; Girard et al., 2005; Graulet et al. 2007). Folate and vitamin B_{12} can be supplied by ruminal synthesis and dietary supply (NRC, 2001). The diet composition and the forage-to-concentrate ratios not only can affect bacterial synthesis of vitamin B_{12}, but also are able to affect the rumen microbial populations. On the other hand, B-vitamin content of feed depends on such factors as climatic conditions, soil properties, species, vegetation stage, and storage and processing (Scott, 1999; Castagnino et al., 2016). Folate naturally is relatively unstable, and therefore folate contents of feeds are variable (Scott, 1999; Ragaller et al., 2008). It is difficult to determine bioavailability of native folates for ruminants (Ragaller et al., 2008).

Choline and Betaine

Choline is found in all mammalian cells and is sometimes referred to as one of the B-vitamins which is a class of water-soluble vitamins, but it does not meet the classic definition of a vitamin (NRC, 2001). Choline can be obtained from the diet or endogenously through the methylation of phosphatidylethanolamine (PE), via the transfer of 3 methyl groups from SAM to PE, to phosphatidylcholine (PC) (Aktas et al., 2014), which is also known as lecithin. Phosphatidylethanolamine N-methyltransferase (PEMT) acts as the key enzyme in the liver, which has the highest PEMT activity (Zinrajh et al., 2014; Zeisel, 2006).

Choline not only plays an important role as a source of methyl groups, like methionine and betaine, for methylation pathways, but also has key functions in the body such as maintaining the integrity of cell structure by phosphatidylcholine synthesis, which is the most abundant phospholipid in mammalian cell membranes (58%; Paoletti et al., 2011), lipid metabolism, and the transport of hepatic lipids via very-low-density lipoprotein (VLDL) synthesis (Chandler and White, 2017). A lack of choline (dietary or endogenously synthesized choline) results in impaired PC biosynthesis, reduced VLDL synthesis and secretion from the liver (Yao and Vance, 1988), and reduced neurotransmission via the neurotransmitter acetylcholine (Picciotto et al., 2012). Within the cell, choline is phosphorylated to phosphocholine or oxidized by choline-oxidase and betaine aldehyde dehydrogenase (in an irreversible reaction) to form the methyl donor betaine (Zeisel and da Costa, 2009). The metabolic pathways of choline, methionine, and folate are interrelated at the point of remethylation of homocysteine to methionine; therefore, when insufficient Met and folic acid are available, choline can operate as an essential nutrient for an alternative pathway for the synthesis of betaine, which has a critical role as a methyl donor (Olthof et al 2005; Zeisel and da Costa, 2009).

Betaine, which is highly water soluble, can be acquired either from the diet or endogenously through the oxidation of choline. Betaine, which serves important roles as a methyl donor for the methylation of homocysteine to form methionine, is demethylated to dimethylglycine (DMG) via betaine homocysteine methyltransferase (BHMT), which is found with high levels of expression in liver and kidney (Ueland et al., 2005; Cho et al., 2006). Following methyl group donation, DMG is converted to sarcosine and glycine via demethylation reactions. Dimethylglycine dehydrogenase (DDH) along with sarcosine dehydrogenase (SDH)

are the enzymes involved in the demethylation processes (Cernei et al., 2013). High concentrations of DMG and SAM can act as inhibitors of BHMT, and this can reduce the utilization of betaine as a methyl donor (Obeid, 2013). Also, the methyl groups from demethylation of betaine end up as 5,10 methylenetetrahydrofolate (Augustin et al., 2016).

Homocysteine

Homocysteine ($HSCH_2CH_2CH(NH_2)COOH$) is a non-protein sulfur-containing amino acid that is predominantly biosynthesized as a product of methionine metabolism in the liver; liver serves as the major organ for the regulation of total homocysteine concentrations in the body (Pajares and Pérez-Sala, 2006). There are different forms of plasma homocysteine: approximately 1% as free thiol; 70-80% as protein-bound homocysteine, which chiefly is linked to albumin by a disulfide bridge; and 20-30% as dimer homocystine or linked with other thiols such as cysteine (Ganguly and Alam, 2015). Serum/plasma total homocysteine is used as a sensitive indicator for assessing folate, vitamin B_{12}, and methyl group deficiency (Green, 2011; Obeid, 2013). Impaired homocysteine metabolism which causes abnormal elevation of plasma total homocysteine is known as hyperhomocysteinemia. Several factors can increase homocysteine concentrations in blood, including: a) genetics; b) age; c) gender; d) dysfunctions in enzymes involved in regulating homocysteine metabolism such as methylenetetrahydrofolate reductase (MTHFR), which converts 5-10 methylenetetrahydrofolate (5,10-methylene-THF) into 5-methylenetetrahydrofolate (5-methyl THF) in the folate cycle, and cystathionine beta-synthase (CBS), which converts homocysteine to cystathionine by transsulfuration pathway; e) vitamin deficiencies such as vitamin B_6 (pyridoxine), vitamin B_{12} (cyanocobalamin), and folic acid, which act as cofactors for homocysteine metabolism in folate cycle and transsulfuration pathway; f) dietary supplements and drugs; and g) some diseases such as pernicious anemia and

renal disease (Obeid, 2013; Malinowska and Chmurzynska, 2009; Maron and Loscalzo, 2009; Welch and Loscalzo,1998). The metabolic fate of homocysteine depends on the two major metabolic pathways: remethylation and transsulfuration.

Remethylation pathway

Homocysteine remethylation to methionine occurs via two pathways: folate pathway (in the most tissues) or betaine pathway (in the liver and kidney) (Selhub, 1999). Approximately 30-50% of Hcy in humans and 50% of Hcy in male rats can be converted through remethylation pathways to methionine (Schwahn et al., 2003).

The remethylation of homocysteine to produce methionine via the folate pathway is dependent on the presence of vitamin B_{12} and folate. Folate after absorption from the small intestine can be converted to 5-methyltetrahydrofolate, tetrahydrofolate (THF), and dihydrofolate (DHF). In the mitochondria, tetrahydrofolate can acquire a one-carbon unit from serine, the major source of one-carbon units for tetrahydrofolate, and be converted to 5,10 methylenetetrahydrofolate (5,10-methylene THF) via serine hydroxymethyltransferase, which is a vitamin B_6-dependent enzyme. In this process, glycine is produced from serine; this is a reversible reaction, such that serine can also be produced from glycine by serine hydroxymethyl transferase. The 5,10-methylene THF can be reduced to 5-methyl THF via methylenetetrahydrofolate reductase in an irreversible reaction. Homocysteine obtains a methyl group from 5-methyl THF to form methionine via methionine synthase, a vitamin B_{12}-dependent enzyme (Amelio et al., 2014; Mato et al., 2008). Because rumen fermentation destroys dietary choline, it is not surprising that in adult ruminants the activity of this pathway is greater than that of the betaine pathway (Pinotti et al., 2002).

The betaine pathway is folate- and vitamin B_{12}-independent, and occurs mainly in the liver and kidney. In this pathway, choline is converted to betaine, which is the direct source of methyl groups for remethylation of homocysteine to Met. Betaine regenerates methionine by donating a methyl group to homocysteine via BHMT. Following methyl group donation, betaine is converted to dimethylglycine (DMG), which in sequence is converted to sarcosine (N-methylglycine) via dimethylglycine dehydrogenase and then to glycine via sarcosine dehydrogenase (Durand et al. 2001; Obeid, 2013).

Transsulfuration pathway

Approximately 50% of Hcy can be degraded through transsulfuration pathway (Imbard et al., 2015). Under normal physiological conditions, the balance between the Hcy production and elimination maintains the homocysteine balance in the body; excessive intake of methionine or protein leads to decreased remethylation of Hcy and increased transsulfuration of Hcy (Nygard et al., 1999). In the transsulfuration pathway, Hcy is degraded in an irreversible pathway that yields cysteine (Cys) and is predominantly found in the liver, kidney, intestine, and pancreas (Malinowska and Chmurzynska, 2009; Brosnan and Brosnan, 2006). Homocysteine reacts with serine (Ser) by the B_6-dependent enzyme cystathionine β-synthase (CBS) leading to cystathionine synthesis (Schweinberger and Wyse, 2016). Cystathionine is then hydrolyzed to Cys by cystathionine γ-lyase (Pan et al., 2012; Nygard et al., 1999; Smacchi and Gobbetti, 1998). Cysteine serves as a precursor for glutathione (GSH, which acts against oxidative stress), proteins, taurine, sulfate, and ammonia (Stipanuk and Ueki, 2011; Smacchi and Gobbetti, 1998).

Consequently, the metabolic pathways of choline, methionine, and folate are related at the point that homocysteine is recycled into methionine; therefore, choline and betaine can spare methionine by increasing the availability of methionine for protein synthesis and

transmethylation pathways. Because methyl donors such as methionine, choline, and betaine are involved in many important functions and biochemical processes in the body, inadequate quantities of methyl groups can either impair transmethylation pathways and/or lead to dysfunction and serious health implications.

Methyl group supplements not only enhance performance in animal production such as dairy cattle (Pinotti, 2012), but also they could theoretically improve the efficiency of N utilization by allowing diets with reduced protein to maintain performance, which would decrease feed costs. The metabolic pathways of choline, methionine, and betaine are closely interrelated (Awwad et al., 2016; Lu et al., 2015; Sun et al., 2011). However, the metabolic requirements of the compounds may be different between ruminants and nonruminants because ruminants have adapted to low choline supply as a result of ruminal degradation (Zhang et al., 2015a; Loest et al., 2002). In the rat, dietary choline supplies 18-54% of the body choline while in ruminant such as sheep nearly all of the choline body pool is supplied by endogenesis synthesis (Robinson et al., 1987). In ruminants, the absorption of methyl groups from the diet is limited because of ruminal destruction (Robinson et al., 1987). The rate of the choline synthesis in sheep liver was lower than rat liver (Neill et al., 1978). In the sheep, liver is responsible for the synthesis of 18% of the endogenous choline body pool (Robinson et al., 1987). Xue and Snoswell (1986) noted that pancreas, in addition to liver, was involved in the transfer of methyl groups in sheep (Xue and Snoswell, 1986). Additionally, the activity of betaine-homocysteine methyltransferase and choline oxidase in sheep liver were less than in rat liver which can limit choline use for methylation reactions. Therfore, the reduction of choline oxidase can be considered as a metabolic adaptation to the low availability of dietary choline in ruminant (Xue and Snoswell, 1986). Creatine synthesis is the major consumer of methyl groups in the body.

More discussion of this will be addressed in the creatine and guanidinoacetic acid section below. Lobley et al. (1996) evaluated methionine flux in response to intravenous infusion of choline plus creatine in sheep. These authors reported that the flux of total methionine was reduced by supplementing creatine in combination with choline. This reduction was reflective of decreased methyl group requirements for creatine synthesis and diminished rates of remethylation of homocysteine to reduce the physiological requirement for methyl groups.

Creatine and guanidinoacetic acid

Overview of creatine

In 1830, Michel Eugene Chevreul, who was a French scientist, extracted a new compound from meat and named it creatine (Greek word “kreas” meaning meat) (Jonquel-Chevalier Curt et al., 2015; Williams et al., 1999). In 1847, Liebig demonstrated that heating creatine with mineral acids led to the loss of one molecule of water and formation of an anhydride he called creatinine (Narayanan and Appleton, 1980). Around the same time, further research by Heintz and Pettenkofer determined that a new substance in urine, a by-product of creatine degradation, was creatinine (Sarikaya et al., 2006). Creatine as a supplement was used for the first time by Soviet athletes during the 1970s in the Olympic Games (Kalinski, 2003). Cells require a constant supply of energy to support cellular processes such as growth and metabolism. Adenosine triphosphate (ATP) is the main and most rapidly available energy source for growth, repair, and maintenance of body cells (Guimaraes-Ferreira, 2014). Creatine (methylguanidine-acetic acid) is known for its essential role in cellular energetics. Creatine is a compound that serves to provide a constant supply of high energy phosphate bonds by allowing the rapid resynthesis of ATP in cells and the maintenance of cellular ATP levels in cells.

Creatine typically is found in higher concentrations in tissues with high energetic requirements such as muscle and brain (da Silva et al., 2009; Wyss and Kaddurah-Daouk, 2009). Creatine can be obtained from the diet from animal-derived foods such as meat and fish (Casey and Greenhaff, 2000) or endogenously synthesized (in humans about 1 g/day) (Cooper et al., 2012) from three essential amino acids (methionine, glycine, and arginine) in the liver, kidney, and pancreas (Baracho et al., 2015; Persky and Brazeau, 2001). Creatine is transferred via the blood through specific creatine transporters to body tissues such as muscle that have high energy demands (Kley et al., 2013). Total creatine includes free creatine plus phosphocreatine (Wyss and Kaddurah-Daouk, 2000). Approximately 95% of creatine (60% phosphocreatine and 40% free creatine) is found in skeletal muscle, with the remaining 5% of creatine situated in brain, liver, kidney, and testes (Bemben and Lamont, 2005; Persky and Brazeau, 2001). The body needs creatine for permanent growth of the muscle (muscle mass development). Especially in growing animals, the requirement for creatine is increased considerably over those of the adults to provide creatine to growing tissues and replace the creatine lost by the breakdown of creatine to creatinine, which is eventually excreted in urine (Brosnan and Brosnan, 2010; Brosnan et al., 2009). In humans, approximately 1.7% of the total creatine pool (2 g/day) is irreversibly converted to creatinine through a spontaneous, non-enzymatic reaction, and the creatinine is then excreted in urine (Riesberg et al. 2016; Da Silva et al. 2014). Therefore, a constant new supply of creatine, which is usually supplied via the combination of diet and endogenous synthesis, is needed to each day replace creatine lost as creatinine (Michiels et al., 2012). The estimated daily average requirement for creatine in humans is approximately 2 g/day, of which about 1 gram is supplied from the diet and the remainder is synthesized endogenously (Ostojic et al., 2013; Cooper et al., 2012). Plants are not a source of creatine, and the body's need for endogenous

synthesis of creatine requires amino acids (arginine, glycine, and methionine) (Murakami et al. 2014). Because plants do not contain creatine, the muscle concentration of creatine is usually lower in vegetarians than omnivores; therefore, the supplementation of creatine may have beneficial effects for vegetarians (Riesberg et al., 2016; Burke et al., 2003). For a 70-kg young male (normal sized person), the creatine pool has been estimated as approximately 120 to 140 g, although it varies between individuals because of differences in muscle mass, skeletal muscle fiber type, and dietary intake (Cooper et al., 2012). As a whole, 1 kg of raw beef contains around 4.5 g of creatine and the typical North American diet contains approximately 1 g/day of creatine (Adhihetty and Beal, 2008).

Creatine as an energy source

As discussed in section, adenosine triphosphate (ATP) is the primary source of energy for cells to maintain growth and normal metabolism. Creatine, due to the energy in phosphate bonds, not only acts as an energy source in tissues with a high energy demand, but also has stimulatory effects on skeletal muscle structure/function, which will be discussed in further detail below.

The availability of ATP is critical for skeletal muscle function such as contraction and relaxation. The concentration of ATP in skeletal muscle has been reported to be roughly 8 mmol/kg wet tissue (Facey et al., 2013). ATP can be generated through three main ways in skeletal muscle to maintain the appropriate ATP/ADP ratio in the cytosol: creatine, glycolysis in the cytosol, and oxidative phosphorylation in the mitochondria (Vander Heiden et al., 2009; Promper et al., 2006). Glycolysis is the major energy provider (ATP) for muscle cells (Alberts et al., 2014). Skeletal muscle utilizes mostly glucose and fatty acids, which are stored as glycogen and triglycerides in skeletal muscles, as sources of energy (Atalay and Hänninen, 2009). In humans, the amount of glycogen found in the skeletal muscle (40–50% of body weight) is

approximately 500 g, whereas glycogen content of the liver is approximately 100 g. The glycogen concentration of liver is greater than of muscle, but the greater overall mass of skeletal muscle than of liver is the main cause for higher glycogen content of the muscle than liver (Jensen et al., 2011). Aerobic metabolism of glucose yields much more ATP than anaerobic metabolism, due to oxidative phosphorylation that depends on using oxygen (Mookerjee et al., 2017).

Phosphocreatine

In 1927, phosphocreatine was discovered for the first time in muscle tissue (Wyss and Kaddurah-Daouk, 2000), whereas creatine kinase (CK) was first described in 1934 by Lohmann (Teixeira and Borges, 2012). Phosphocreatine, which is critical for maintenance of energy in cells, is generated by the transfer of a phosphate group from ATP to creatine via creatine kinase (CK) in a reversible reaction resulting in release of ADP (Guimaraes-Ferreira, 2014). A high ratio of ATP to ADP in the cell when the ATP consumption rate is low leads to phosphocreatine synthesis through transfer of a phosphoryl group from ATP to creatine via creatine kinase (Wyss and Kaddurah-Daouk, 2000).

ADP + creatine-phosphate ↔ ATP + creatine + H^+ (Hopwood et al., 2006).

The fast-twitch skeletal muscles contain large amounts of phosphocreatine and high levels of CK activity; therefore, they have strong potential for energy generation during very intense activities, which need a quick energy boost for a limited time period (Okumura et al., 2005; Wyss and Kaddurah-Daouk, 2000). Fast-twitch muscles have higher potential energy production via anaerobic pathways (phosphocreatine and glycogen degradation) (Casey and Greenhaff, 2000) than slow twitch muscle which has higher aerobic energy creation. The muscle

contraction occurs as a result of ATP consumption during muscular activity; therefore, the muscle is unable to contract when the ATP level is low due to muscular activity consuming the available ATP (Guimaraes-Ferreira, 2014). Muscle cells store a very small quantity of ATP; therefore, during rapid muscle consumption of ATP, other mechanisms must exist to supply energy. During severe exercise and sudden increases in ATP demand, ATP can be produced from phosphocreatine faster than from glycolysis (Chikani and Ho, 2013; Casey and Greenhaff, 2000). In these cases, 70% of ATP production is from phosphocreatine (Sahlin, 2014).

Creatinine biosynthesis

Creatinine is the end product of phosphocreatine and creatine metabolism through an irreversible nonenzymatic reaction, and it is eliminated by the kidneys and passed out in the urine (Brosnan and Brosnan, 2016). The excretion rate of creatinine and the body's pool of total creatine (phosphocreatine plus free creatine) are both relatively constant and proportional to each other (Clark and Cecil, 2015). In vivo data show that approximately 2.6%/day of endogenous creatine (rate of production) and 1.1%/day of phosphocreatine are converted by an irreversible process to creatinine (Cooper et al., 2012). The average conversion rate of total creatine into creatinine was estimated to be roughly 1.7%/day (Wyss and Kaddurah-Daouk, 2000). Serum creatinine can be an inexpensive and reliable biomarker for estimation of skeletal muscle mass in patients with chronic kidney disease (Patel et al., 2013). Previous studies have shown creatine supplementation increased not only creatine pool size and serum creatinine concentration, but also reduced endogenous creatine synthesis (Ostojic et al., 2013; Persky and Brazeau, 2001).

Creatine and bodyweight

The popularity of creatine supplementation did not begin until the early 1990s as a way for athletes to improve their performance (Buford et al., 2007). In recent years, there has been widespread use of creatine as a dietary supplement among athletes to maximize their exercise performance (Racette 2003). In the United States, a large portion of $2.7 billion spent on sport nutrition supplements is from the sale of creatine supplements (Jager et al., 2011). The sales of creatine were estimated at US $100 and $400 million in 1998 and 2001, respectively (Paddon-Jones et al., 2004; Bird, 2003).

Creatine as a nutritional supplement has the ability to positively influence muscle mass and body weight in humans (Gualano et al., 2012; Hopwood et al., 2006). Numerous studies have demonstrated that body mass increased significantly after creatine supplementation (Cooper et al., 2012).

Some factors such as sodium, which acts as creatine cotransporter into skeletal muscle, and insulin, which enhances muscle creatine uptake by stimulating muscle blood flow and thereby making more creatine available to muscles, can have influence on creatine absorption and transport into skeletal muscle (Jager et al., 2011; Brault and Terjung, 2003; Steenge et al., 1998). Schedel et al. (2000a) illustrated that 6-day administrations of high doses of creatine (30 g/d) to athletes, who were randomized into a control group (n = 5) and experimental group (n = 5), significantly enhanced serum and urine creatine concentrations and serum creatinine concentrations following creatine administration. Athletes who were supplemented with creatine also showed significant increases of >1.5 kg in body weight, even though there were no changes to percent body fat. Creatine supplementation increased the ratio of urine/serum creatine concentration from 24-fold before supplementation to 239-fold after supplementation.

Williams (2006) reported that short-term creatine supplementation could enhance weight gain, although it was suggested that enhanced weight gain after treatment with creatine might be associated with: 1) an increase in protein synthesis by the cell swelling which serves as a stimulatory signal for protein synthesis (Paddon-Jones et al., 2004), 2) a decrease in muscle atrophy, or 3) tissue water retention because of osmotic properties of creatine (Moon and Cobbold, 2016; Vojvodic-Ostojic, 2015). Kreider (2003) suggested that long-term creatine supplementation might also result in increased lean tissue mass without increased total body water, muscle creatine, or phosphocreatine content. It is important to note that the increasing body mass may not be only due to increased water retention because water retention might be a sign of protein retention (Schedel et al., 2000b). In addition, numerous research studies on creatine supplementation indicated significant enhancement in body mass gain (1 to 2 kg) after one week of creatine supplementation (Buford et al., 2007). Also, combining creatine with carbohydrate is described as a promising strategy to maximize cellular creatine uptake; therefore, several research studies have been conducted to evaluate whether co-ingesting creatine with carbohydrates may help creatine uptake into muscle. Green et al. (1996 a,b) studied creatine supplementation (5 g) and carbohydrate ingestion (95 g) in healthy male humans, and they observed a greater increase in muscle creatine uptake (60%) and retention of creatine with lower levels of creatine in the urine when carbohydrates were supplemented. This response might be related to the elevated insulin secretion following carbohydrate ingestion. Schedel et al. (2000b) demonstrated that the administration of 20 g creatine monohydrate to healthy men over 6 hours could significantly increase growth hormone secretion, which might result from the relationship between the elevated intracellular creatine concentration and growth hormone secretion. Growth hormone is secreted by the anterior pituitary via cyclic adenosine monophosphate (cAMP) which

is intracellular signal transduction produced by the hydrolysis of adenosine triphosphate (ATP) by adenylate cyclase activity.

In an experiment reported by James et al. (2002), 320 pigs were assigned to 1 of 4 treatments for 30 d (control group; 3 g/d creatine monohydrate (CMH); 25 g CMH/d for 5 d followed by 3 g CMH/d for the next 25 d; 25 g/d CMH for 5 d before slaughter) in a randomized complete block design. They found no effect of treatment on finishing pig growth performance; however, creatine supplementation helped to improve the firmness of longissimus muscle. Previous research has shown that creatine supplementation increased bone mineral density in rats; it has therefore been suggested that creatine supplementation can be considered in elderly males with osteopenia and osteoporosis to reduce bone loss (Moon and Cobbold, 2016). In an experiment reported by Zhang et al. (2014), 320 Arbor Acres male broiler chickens (28-day-old) received three levels of creatine: 0 (160 birds), 600 (80 birds), or 1200 mg/kg (80 birds) creatine monohydrate for 14 days before slaughter. The results showed no effect of treatment on growth performance and carcass traits of stressed broilers; however, 1,200 mg/kg dietary creatine significantly reduced live weight loss, muscle glycolytic potential, and muscle lactate accumulation in transported broilers, which can help to maintain meat quality characteristics. Carvalho et al. (2013) fed vegetable diets based on corn and soybean meal supplemented with or without animal meal (5% blood meal or 5% meat and bone meal) and creatine (600 g/ton) to 1,080 one-day-old male chicks until 42 days of age. They observed no effect of creatine supplementation with or without meat and bone meal on performance or carcass yield of broilers, but creatine supplementation increased broiler body weight of birds fed blood meal at 42 days of age.

Guanidinoacetic acid (GAA)

In 1950s, the historical clinical studies, which were the first reports on GAA effects, demonstrated that dietary GAA intake has significant impact on human physiology (Ostojic, 2015b). Research on GAA, which began approximately 80 years ago, showed that GAA can be an important component in human metabolic processes (Ostojic, 2015b). Previous research had reported the presence of GAA in urine of normal humans, rats, and dogs. However, the muscle, heart, or liver of the rat did not show the presence of GAA (Thomas, 1938). Guanidinoacetic acid (glycocyamine or guanidinoacetate; $C_3H_7N_3O_2$) is a nitrogenous organic acid which can be obtained from dietary sources or mainly synthesized endogenously in the kidney from the amino acids glycine and arginine (Ostojic, 2015a). Guanidinoacetic acid has generated particular interest because of its beneficial effects on human body such as cellular bioenergetics and also acting as creatine precursor (Ostojic et al., 2013a). Guanidinoacetic acid, which has the replenishing capacity of creatine, can be used in the lack of creatine production to replenish the body's creatine and ATP stores. Therefore, supplementation of GAA can be considered as an alternative source for energy production (body's ATP stores) (Ostojic, 2015a).

Guanidinoacetic acid has shown various physiological functions such as neuromodulation and stimulation of hormone (notably insulin) secretion. GAA is more potent than arginine or creatine to increase insulin secretion (Ostojic, 2014). Furthermore, GAA production consumes a large amount of arginine (Brosnan et al., 2011), thus GAA supplementation is able to spare arginine by reducing GAA synthesis, which results in sparing of arginine for other bodily functions, such as protein metabolism, nitric oxide production, and hormonal release (Ostojic, 2015b).

Stability and bioavailability of creatine and guanidinoacetic acid

Creatine has low solubility and stability in water because of intramolecular cyclization. Although, it should be noted that there are factors such as temperature and pH that may affect the solubility of creatine, using a solution with a low pH and/or increasing the temperature can enhance the solubility of creatine (Jager et al., 2011). Importantly, GAA can be a more suitable feed additive compared with creatine due to it being more stable and bioavailable as well as less expensive (approximately 40%) than creatine (Ostojic et al., 2017; Ostojic and Vojvodic-Ostojic, 2015; Ostojic et al., 2015). The greater thermal stability and insolubility of GAA in water than creatine is correlated with the influence of the additional methyl group on the creatine molecule (Ostojic et al., 2017). GAA tends to dissolve in polar solvents such as water because of formation of hydrogen bonds and electrostatic interaction between carboxyl group and guanidino group by strong coulomb interactions. However, the preparation techniques and ingestion have been demonstrated to highly influence GAA bioavailability (Vraneš et al., 2017). Plasma creatine levels after oral creatine supplementation can be used as an indicator of change of creatine bioavailability; however, the changes in levels of total plasma creatine cannot directly provide an estimate of a potential increase in achieving the desired performance (Jager et al., 2011). In a recent research, Speer et al., (2020) evaluated the relative bioavailability of GAA between abomasal (10 or 20 g/d GAA) and ruminal infusion (10 or 20 g/d GAA) in cattle. The authors reported that the ruminal bioavailability of GAA based on plasma creatine and urinary creatine concentrations were 47% and 49%, respectively, suggesting the effectiveness of ruminal infusion of GAA based on increasing plasma and urinary concentrations of creatine can be approximately 50% of post-ruminal GAA supplementation; therefore, use of GAA as a ruminant

feed additive would require either approaches to increase protection from ruminal digestion or twice the intended dose to achieve desired outcomes (Speer et al. 2020).

In addition, plasma creatine can be elevated by reducing creatine uptake by target tissue (Jager et al., 2011). The bioavailability of creatine to support the target tissue such as muscle can be estimated through muscle biopsy and/or whole body creatine retention. The efficiency of creatine absorption, response to creatine supplementation, and whole body creatine retention may be affected by different forms of creatine (Jager et al., 2011). Additionally, several studies have demonstrated that muscle creatine and carbohydrate storage can be augmented through combining high levels of glucose (95 g) and creatine (5 g) and also a combination of creatine (5 g), carbohydrate (47–97 g), and protein (50 g) can increase creatine retention in humans (Jager et al., 2011).

Interactions between guanidinoacetic acid and creatine

Endogenous synthesis of creatine mainly occurs in the liver and kidney, although it can also be synthesized at a small rate by the pancreas (Riesberg et al., 2016; Wyss and Kaddurah-Daouk, 2000), involving 3 amino acids (arginine, glycine and methionine) and two enzymes (arginine:glycine amidinotransferase (AGAT) and guanidinoacetate methyltransferase (GAMT)) by a two-step mechanism (Brosnan and Brosnan, 2004). The first reaction takes place mainly in the kidney, wherein the transfer of an amidino group from arginine to the amino group of glycine by AGAT forms ornithine and GAA. Guanidinoacetic acid then enters into blood to be absorbed by the liver for the subsequent steps of creatine synthesis. In the second reaction, a methyl group from S-adenosylmethionine in an irreversible reaction catalyzed by GAMT is transferred to GAA to form creatine and S-adenosylhomocysteine (SAH). Creatine is then released into the blood stream to be taken up by various tissues, but mainly by muscle cells (Riesberg et al., 2016;

Wyss and Kaddurah-Daouk, 2000). Interestingly, muscle, which has the highest level of creatine, contains a very small amount of AGAT and GAMT (Brosnan and Brosnan, 2010). The highest level of AGAT activity is found in the kidney and the pancreas, whereas GAMT has highest activity in the liver and intermediate activity in the pancreas (Brosnan et al., 2009); therefore, GAA can be made by the kidney and pancreas (Ostojic et al., 2014). More methyl groups derived from SAM are used for the methylation of GAA to creatine than for other methylation reactions (approximately 40–75% of all methyl groups derived from SAM: Peters et al., 2015; Ostojic, 2014). A reduction in AGAT enzyme activity was found in rat kidney following an increase in serum concentrations of creatine (inhibitory effects of creatine on AGAT) (McBreairty et al., 2013); therefore, the levels of creatine can have a role in the regulation of renal GAA production (Edison et al., 2007).

Guanidinoacetic acid (GAA) supplementation: Supplemental GAA and homocysteine, GAA, creatine and creatinine concentrations in monogastric animals and humans

Monogastric animals

As discussed in the previous section, more methyl groups of SAM are consumed by GAA for creatine synthesis than all other methylation reactions; therefore, GAA supplementation may not only result in reduced methyl group availability and increased methyl group demand, but also can affect homocysteine metabolism by increased conversion of SAM to SAH in the body (Ostojic, 2014a). Administering GAA can raise plasma homocysteine concentrations. Stead et al. (2001) examined the effect of 2 weeks of 3.6 g/kg GAA supplementation on plasma homocysteine concentration in male rats. Elevation of plasma homocysteine concentration

(~50%) was observed after treatment with GAA, which indicated higher demand for the methylation of GAA. Also, when isolated rat hepatocytes were incubated with methionine in the presence of GAA, the homocysteine export was significantly increased by 47% compared with methionine alone. They suggested that the administration of GAA by 5 g/kg for 5–10 days can increase homocysteine synthesis.

Following 10 days of five levels of GAA supplementation (0, 2.5, 5, 7.5, and 10 g/kg) on six-week-old male rats (120–140 g), Fukada et al. (2006) reported increases in plasma total homocysteine concentration for 5 and 10 g/kg GAA by 255 and 421%, respectively. This might be due to a reduction in homocysteine remethylation. These researchers also showed that, following GAA supplementation, hepatic S-adenosylmethionine (SAM) concentration decreased, whereas liver had higher concentrations of homocysteine and S-adenosylhomocysteine (SAH). These may be a result of increased conversion of SAM to SAH during methylation of GAA as well as reduced cystathionine beta-synthase (CBS) activity, which could reduce cystathionine formation from homocysteine. It was also suggested that there is an association between SAM and CBS activity because a reduction of CBS activity was observed when SAM concentration was decreased in liver.

In rodents, the administration of GAA with or without the addition of methyl donors such as choline or betaine showed that choline in combination with GAA could reduce the increased hepatic SAH and homocysteine concentration induced from the intake of GAA. Also, the intake of GAA alone increased plasma homocysteine concentration whereas the increase of plasma homocysteine concentration, induced by GAA, was suppressed by supplementation with choline and betaine in hyperhomocysteinemic rats (Setoue et al., 2008).

Murakami et al. (2014) fed quail breeders corn-soybean meal basal diets with different dietary levels of guanidinoacetic acid (0.00, 0.06, 0.12, 0.18, and 0.24%) and showed elevated GAA, creatine, and creatinine content of the egg with increasing dietary levels of GAA, suggesting that GAA supplementation increased GAA transportation from the blood stream into the liver to be converted into creatine to improve creatine transfer into the egg.

In piglets allotted to six dietary GAA concentrations (0, 600, 900, 1,200, 4,500 or 6,000 mg GAA/kg feed), GAA supplementation increased plasma GAA for 4,500 and 6,000 mg GAA/kg supplementation, and plasma homocysteine increased following the level of GAA supplementation between 1200 and 6000 mg GAA/kg feed (European Food Safety Agency, 2016).

Tossenberger et al. (2016) demonstrated that broiler chicks, which were fed two levels of GAA (0.6 g and 6.0 GAA per kg of feed) from day 1 to 35 (starter and grower period), had not only greater plasma and urinary levels of creatine and creatinine by supplementation of 6.0 g/kg GAA, but also an elevated plasma homocysteine level was observed for the greater dose of GAA. The observed increase in plasma homocysteine was attributed to the increased demand for methyl groups to convert GAA to creatine, which could result in increased homocysteine production and reduced homocysteine remethylation due to a deficiency of methyl groups. Additionally, there were increases in urinary excretion of GAA (3.37 to 102.62 mg/kg$^{0.75}$ daily), creatine (1.07 to 29.43 mg/kg $^{0.75}$ daily), and creatinine (3.40 to 66.97 mg/kg $^{0.75}$ daily) by increasing levels of supplemental GAA from 0 to 6.0 g GAA/kg, respectively, suggesting that the renal excretion is the main route of elimination of excess GAA and its metabolites creatine and creatinine in chicks.

Lemme et al. (2007a) fed male broilers four dietary GAA concentrations (0, 0.20, 0.40, or 0.60 g/kg GAA) in a vegetable-based diet. Results indicated increased muscle creatine by about 14% with increasing GAA supplementation levels, suggesting GAA can act as a source of creatine.

Humans

The study, involving twenty-four young healthy volunteers (12 men and 12 women), was designed to investigate the effect of a single oral dose of guanidinoacetic acid (2.4 g) on serum total homocysteine concentration. Results showed that serum total homocysteine increased up to 40% after administration of the oral GAA (Ostojic et al., 2014b).

A study by Ostojic et al. (2013b) investigated the effect of oral supplementation of GAA (2.4 g/d) in combination with and without methyl group donors for 8 weeks to healthy human (20 male and female volunteers). There was a significant increase in serum and urinary GAA, creatine, and total plasma homocysteine but no effect on serum and urine creatinine by administration of 2.4 g of GAA. These data also suggested that GAA-induced hyperhomocysteinemia from GAA supplementation can be prevented by administering the GAA in combination with methyl donors.

Considering studies on GAA, Ostojic et al. (2014c) evaluated the effects of three different dosages of GAA (1.2, 2.4 and 4.8 g/day) in 48 healthy humans for 6 weeks on serum and urinary variables. Results of this study indicated that the addition of GAA significantly increased serum and urinary GAA, creatine, and creatinine compared with the placebo. Although, serum GAA and creatine were significantly greater in participants receiving the highest dose of GAA compared to other GAA dosages. There was also a significant increase in

plasma total homocysteine (T-Hcy) following GAA supplementation. It has been suggested that the exogenous GAA intake has the potential to increase creatine production, although during this process homocysteine formation can also increase and this reflects an elevation of methyl group requirements.

Ostojic (2016a) observed that the administration of 3 g/d of oral GAA for 4 weeks to four healthy young men increased total creatine levels by 15.9% in the skeletal muscle. These results suggested that the increases in muscle creatine might relate to increased export of creatine synthesized from liver (because of increased in GAA supply), endogenous creatine synthesis by muscle, or both.

Ostojic et al. (2014b) demonstrated increased serum and urinary GAA and creatine concentrations with oral administration of one dose of GAA (2.4 g) in young healthy volunteers, suggesting that increases in serum and urinary GAA could be attributed to increases in GAA absorption and the renal elimination of excess non-methylated GAA that was not converted into creatine. It should be noted that the creatine transporters play an important role in the distribution of body creatine and also act as a clearance mechanism for creatine via increases in creatine uptake by skeletal muscle. Beside creatine transporters, there are other factors affecting the removal of creatine such as renal creatine excretion and creatinine formation. Creatine pharmacokinetics are probably affected by some factors such as dietary components, disease, and age as well (Persky et al., 2003). Also, there is a limited capacity to store creatine in the tissues; therefore, muscle cannot be overloaded by increasing circulating creatine (DeGroot et al., 2017).

Supplemental GAA and livestock performance

Heger et al. (2014) investigated the effects of GAA supplementation of 0.6 g/kg with different levels of metabolizable energy on growth performance and carcass traits. Results showed that gain:feed was increased by GAA supplementation. Other researchers also have observed improvements in efficiency of gain (Metwally et al., 2015; Dilger et al., 2013; Michiels et al., 2012; Lemme et al., 2011; Lemme et al., 2007a; Ringel et al., 2007) when GAA was supplemented. Heger et al. (2014) also reported that GAA supplementation reduced feed intake, suggesting that the reduction in feed consumption could be due to effective utilization of energy or negative consequences of GAA consumption, but Ringel et al. (2007) found no effect of GAA on feed intake in broilers. In contrast, a study by Tossenberger et al. (2016) demonstrated no impact of GAA supplementation on feed conversion ratio in broiler chicks.

Michiels et al. (2012) studied the effects of two doses of supplemental GAA (0.6 or 1.2 g of GAA per kilogram of feed) in the diet containing corn-soybean-based (negative control) in comparison with a diet containing fishmeal (positive control diet). The results indicated that GAA supplementation enhanced breast meat yield (30.6%) compared with negative control (29.4%) and positive control birds (30.2%). The increases in performance and breast meat yield in chickens by GAA supplementation were consistent with the responses observed by Ringel et al. (2008a,b), suggesting the significant effect of GAA supplementation observed during the finisher and growing periods in broilers was because of increased the availability of arginine for protein synthesis due to arginine-sparing effect of GAA (Ostojic, 2015b).

Esser et al. (2017), working with male broiler chicks which were under high environmental temperature and received a vegetable diet based on corn and soybean meal supplemented with GAA (0.08%) and L-arginine (0.8%), showed that, although both GAA and

arginine supplementation resulted in a decrease of abdominal fat deposition and increased carcass weight and breast yield compared to the control diet and the control diet containing meat meal, GAA supplementation was more advantageous than arginine due to greater meat yield improvement and greater affordability. These results agreed with work by Metwally et al. (2015) who observed that GAA supplementation in broiler diets not only increased growth performance such as weight gain and breast meat yield, but also increased protein content of the breast muscle and decreased abdominal fat deposition during the growing period. Similarly, Lemme et al. (2011) showed that GAA supplementation in broiler diets increased body weight (gain) and muscle creatine content. This is in line with the report by Liu et al. (2015) who indicated that not only the concentrations of creatine in longissimus dorsi of finishing pigs was significantly increased with dietary supplementation of GAA, but also supplemental GAA could improve meat quality. Wang et al. (2012) also observed improved meat quality in growing-finishing pigs fed a diet based on maize-soybean meal with four levels of GAA (0.0, 0.8, 1.2, 2.0 g/kg), although there was no significant effect of GAA on growth performance.

Lemme et al. (2007b) compared the administration of different dietary levels of guanidinoacetic acid (0, 0.06, 0.12, 0.18, and 0.24%) when plant-based diets containing 6% meat meal and bone meal were fed to Ross broilers (1056 male and 1056 female broilers). These researchers reported that the 0.06% supplemental GAA improved gain:feed and body weight gain of female broilers. Also, 0.06% and 0.12% GAA supplementation enhanced breast meat yield in male and female broilers, respectively. These results suggest that dietary supplementation of GAA can improve broiler performance, and the optimal supplementation level of GAA is between 0.06% and 0.12%, with an average 0.07%.

Michiels et al. (2012) also indicated that dietary supplementation of 0.6 and 1.2 g/kg of GAA increased breast meat yield and creatine concentration in breast muscle by 11.1 and 15.7%, respectively, suggesting that the increases in creatine contents in breast meat could be associated with the effective conversion of GAA into creatine. In addition, supplying 1.2 g/kg of GAA significantly enhanced insulin-like growth factor I (IGF-I). Overall, IGF-I performs specific functions inside of the body such as tissue growth and development, insulin-like activity, and proliferative and anti-aging effects (Puche and Castilla-Cortazar, 2012). A study by Burke et al. (2008) assessed the effects of 0.25 g creatine/kg lean-tissue mass for 7 days or 0.06 g creatine/kg lean-tissue mass for 49 days on insulin-like growth factor-I (IGF-I) content in muscle of male and female participants under high-intensity exercise. They reported improved intramuscular IGF-I concentration (78%) compared with placebo (55%) in response to creatine supplementation and resistance training in men and women. It is important to note that the insufficient intake of essential amino acids can reduce IGF-I production; therefore, essential amino acids are needed to support maximum IGF-I production (Burke et al., 2008). These findings showed that performance and carcass characteristics are improved in all-vegetable diets with supplemental GAA. In addition, Alsever et al. (1970) observed the increases in plasma insulin level following GAA infusion in the isolated rat pancreas and also indicated that GAA is more effective than arginine or creatine in stimulating insulin release; also, GAA showed that it has potential to reduce plasma glucose through improved insulin sensitivity (Ostojic, 2015b), suggesting that the polarity property of GAA, which can cause the depolarization of the pancreatic islet cell membrane, might lead to stimulating the release of insulin. A variety of reasons may affect insulin secretion such as changes in the flux of sodium and potassium ions

across the pancreatic islet cell membrane, and the activation of protein kinase A and C (Ostojic, 2015b).

Murakami et al. (2014) fed quail breeders corn-soybean meal basal diets with different dietary levels of guanidinoacetic acid (0.00, 0.06, 0.12, 0.18, and 0.24%) and showed improvement in both weight gain and feed conversion, resulting in overall improved fertility and postnatal progeny performance and reduced embryo mortality. High energy is a requirement for sperm motility; therefore, creatine has potential to enhance sperm motility and speed (Murakami et al., 2014).

The European Food Safety report (2016), which summarized the results of several trials evaluating GAA effects, indicated that the safe GAA intake level for effectiveness, fattening, and improving the performance in chickens and piglets is between 600-1200 mg GAA/kg if methyl donors are adequately supplied in the diet. The report also specified that GAA was not expected to present any risk to the environment. The improvement in hatchability for 2 levels of GAA (800 and 1,200 mg GAA/kg feed) in chickens allotted to five dietary GAA (0, 400, 800, 1,200 or 1,600 mg GAA/kg feed) was observed as well (European Food Safety Agency, 2016).

In a study conducted by Lemme at al. (2010), 576 male Ross broilers received GAA (0.8 GAA/kg feed) or creatine monohydrate (1.0 creatine/kg feed) in a methionine-deficient plant diet or the same diet supplemented with methionine (0.37% vs. 0.57%, respectively). Results indicated that both GAA and creatine supplements improved feed:gain but did not affect weight gain and breast meat yield when dietary methionine was sufficient. In contrast, for broilers fed the methionine-deficient diet, GAA and creatine supplementation were ineffective which showed

that taking both GAA and creatine supplements can be more effective when the availability of methionine is not limiting.

Teixeira et al. (2017) also examined the effects of different levels of guanidinoacetic acid on performance and blood creatinine in 90 barrow piglets from 21 to 63 days old. Results showed no significant difference in performance (daily weight gain, daily feed intake, or feed conversion) and blood creatinine.

In a recent study, Li et al. (2020) evaluated effects of increasing levels of GAA: 0 (control) , 0.3 (low-GAA), 0.6 (medium-GAA) and 0.9 (high-GAA) g/kg DM in a corn-alfalfa based diet on 48 Angus bulls (11.8 ± 0.3 months of age and 391 ± 11.6 kg of BW) for 90 days. The results indicated that DMI, average daily gain (ADG), body weight (BW), and feed conversion ratio (FCR) improved when GAA was supplemented; however, maximal responses to GAA were achieved at high and medium level of GAA for DMI, ADG, BW and low-GAA for FCR at 60 and 90 days. These authors stated that increases in ADG and BW might be due to the improvement in intestinal morphology (Amiri et al., 2019), resulting in elevation of DMI and nutrient degradation by supplemental GAA. Additionally, the greater increase in digestibility of DM, OM, NDF, and ADF by 0.9 g/kg DM supplemental GAA were observed. While there were no differences in blood Hcy, arginine and Met by administration of GAA, an increase in blood creatine concentration was observed when cows were supplemented with GAA, particularly high level of GAA led to the greatest increase in blood creatine concentration (Li at al., 2020). Therefore, authors suggested that GAA might improve DMI by increasing nutrient digestibility, ruminal total VFA concentration, and blood creatine concentration (Li at al., 2020), which can stimulate food intake (Galbraith et al., 2006). It has also been observed a decrease in serum L-arginine glycine amidine transferase (AGAT) and guanidine acetate N-methyltransferase

(GAMT) levels when bulls received dietary GAA than control group, indicating the inhibitory effect of supplementary GAA on AGAT activity and S-adenosylhomocysteine (SAH) on GAMT (Li at al., 2020).

Supplemental GAA vs. creatine

McBreairty et al. (2015), studying the effects of GAA and creatine loading on tissue creatine stores for 18 to 19 d in Yucatan miniature pigs, found that hepatic creatine concentrations were significantly higher for creatine- and GAA-supplemented groups compared to the control group; GAA supplementation led to ~2-fold higher hepatic creatine than creatine supplementation. Furthermore, utilization of GAA increased muscle creatine (~20%) relative to the control and creatine groups and significantly increased plasma creatine concentrations (~70%) compared to the control group. These authors suggested that GAA supplementation was more effective in increasing in muscle and liver compared to creatine supplementation in Yucatan miniature pigs. In addition, results showed the hepatic SAM concentration in pigs receiving GAA supplementation was lower than the control and creatine groups; however, hepatic methionine and arginine did not differ among groups.

Stead et al. (2001) demonstrated that GAA and creatine supplementation increased muscle creatine stores and plasma creatine levels. Muscle total creatine was significantly higher by 49% in male rats receiving dietary creatine (3.6 g/kg) and by 39% in those receiving dietary GAA (4 g/kg) compared with control group; this showed that, contrary to previous research, creatine supplementation was more effective than GAA supplementation to improve total muscle creatine content.

Ostojic et al. (2016b) compared the effectiveness of GAA (3 g/day) versus creatine (3.4 g/day) on muscle creatine levels in healthy men for 4 weeks and demonstrated that both GAA

and creatine supplementation could elevate the tissue creatine levels, but GAA was more efficient and effective at increasing muscle creatine levels by 16.2% compared to creatine.

Conclusion

Methionine acts as a methyl donor and typically is a limiting amino acid for beef and dairy cows in order to achieve optimum performance. Methionine also has important functions in protein synthesis, increasing growth rate and the efficiency of N usage. Therefore, to ameliorate the methionine deficiency, supplementation of rumen-protected methionine, to limit ruminal bacterial degradation of dietary methionine before it passes to the small intestine for absorption, is a common method to improve the methionine deficiency in ruminant diets. Creatine supplementation is viewed as a means of increasing muscle mass and lean growth rate in humans. Creatine serves as an energy source and is synthesized in the body from guanidinoacetic acid (GAA). Therefore, it should be considered that GAA supplementation, under conditions where methyl groups are not limiting, can be an effective way to increase creatine supply to the body to increase muscle development and nitrogen retention. It is important to note that improved N use efficiency can be not only a cost-effective method for improving animal performance, but also can decrease the negative impact of excessive N on the environment. Overall, the growth-enhancing effect of GAA supplementation indicates that GAA has the potential to improve nitrogen utilization efficiency and the growth rate performance of cattle when methionine supply is adequate. Further work will be needed to verify this effect.

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Chapter 2 - Effect of post-ruminal guanidinoacetic acid supplementation on creatine synthesis and plasma homocysteine concentrations in cattle[1]

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Abstract

Creatine stores high-energy phosphate bonds in muscle, which is critical for muscle activity. In animals, creatine is synthesized in the liver from guanidinoacetic acid (**GAA**) with methylation by *S*-adenosylmethionine. Because methyl groups are used for the conversion of GAA to creatine, methyl group deficiency may occur as a result of GAA supplementation. With this study, the metabolic responses of cattle to post-ruminal supplementation of GAA were evaluated with and without methionine (**Met**) supplementation as a source of methyl groups. Six ruminally cannulated Holstein heifers (520 kg) were used in a split-plot design with treatments arranged as a 2 × 5 factorial. The main plot treatments were 0 or 12 g/d of L-Met arranged in a completely randomized design; three heifers received each main plot treatment throughout the entire experiment. Subplot treatments were 0, 10, 20, 30, and 40 g/d of GAA, with GAA treatments provided in sequence from lowest to highest over five 6-d periods. Treatments were infused continuously to the abomasum. Heifers were limit-fed twice daily a diet consisting of (dry matter basis) 5.3 kg/d rolled corn, 3.6 kg/d alfalfa hay, and 50 g/d trace-mineralized salt. Plasma Met increased ($P < 0.01$) when Met was supplemented, but it was not affected by supplemental GAA. Supplementing GAA linearly increased plasma arginine (% of total amino acids) and plasma concentrations of GAA and creatinine ($P < 0.001$). Plasma creatine was increased at all levels of GAA except when 40 g/d of GAA was supplemented with no Met (GAA-quadratic × Met, $P = 0.07$). Plasma homocysteine was not affected by GAA supplementation when heifers received 12 g/d Met, but it was increased when 30 or 40 g/d of GAA was supplemented without Met (GAA-linear × Met, $P = 0.003$); increases were modest and did not suggest a dangerous hyperhomocysteinemia. Urinary concentrations of GAA and creatine were increased by all levels of GAA when 12 g/d Met was supplemented; increasing GAA

supplementation up to 30 g/d without Met increased urinary GAA and creatine concentrations, but 40 g/d GAA did not affect urine concentrations of GAA and creatine when no Met was supplemented. Overall, post-ruminal GAA supplementation increased creatine supply to cattle. A methyl group deficiency, demonstrated by modest increases in plasma homocysteine, became apparent when 30 or 40 g/d of GAA was supplemented, but it was ameliorated by 12 g/d Met.

Key words: creatine, guanidinoacetic acid, homocysteine, methionine

Abbreviations: AA, amino acid; CP, crude protein; DM, dry matter; GAA, guanidinoacetic acid; HPLC, high-performance liquid chromatography; Met, methionine; NDF, neutral detergent fiber; TDN, total digestible nutrients.

Introduction

Guanidinoacetic acid (**GAA**) is a precursor for creatine, which is a compound that allows the storage of high-energy phosphate bonds in muscle (Brosnan et al., 2009; Murakami et al., 2014). Creatine can be supplied through the diet or synthesized endogenously (Brosnan and Brosnan, 2007). Production of GAA occurs via the transfer of an amidino group from arginine to glycine, largely in the kidney (Brosnan and Brosnan, 2004). A methyl group from *S*-adenosylmethionine is transferred to GAA to form creatine, predominantly in the liver; this reaction is catalyzed by guanidinoacetate *N*-methyltransferase. Creatine is released to the blood to be taken up by a tissue such as skeletal muscle. In skeletal muscle, the guanidino group of creatine can accept a phosphate group from adenosine triphosphate to produce adenosine diphosphate and phosphocreatine. Creatinine is a spontaneously produced end product of creatine and creatine phosphate, and it is excreted in the urine (Wyss and Kaddurah-Daouk, 2000).

Methionine (**Met**) is an essential amino acid (**AA**) that is often limiting for cattle (Schwab et al., 2004; Varga, 2010). Activation of Met forms *S*-adenosyl-L-methionine which is the body's primary methyl group donor. Homocysteinemia can result from conditions where methyl groups are limiting (Brosnan and Brosnan, 2006; Williams and Schalinske, 2007). Because GAA is an obligate consumer of methyl groups, it may produce a methyl group deficiency (Williams and Schalinske, 2010). Some compounds that act as methyl donors, such as Met, choline, and betaine, may prevent methyl group deficiency and the associated homocysteinemia (Tehlivets et al., 2013).

Our hypotheses were that 1) GAA supplementation could create a methyl group deficiency in cattle and 2) Met supplementation could prevent the methyl group deficiency.

Materials and Methods

All experimental procedures involving cattle were approved by the Institutional Animal Care and Use Committee at Kansas State University.

Six ruminally cannulated Holstein heifers (520 ± 49 kg initial body weight) were used in a 44-d experiment, composed of 14 d for adaptation to facilities and diet and five 6-d periods, with samples collected on days 3 and 6 of each period. The experiment used a split-plot design. The main plot treatments were 0 or 12 g/d of L-Met arranged in a completely randomized design; three heifers received each main plot treatment throughout the entire experiment. Subplot treatments were 0, 10, 20, 30, and 40 g/d of GAA, with treatments provided in sequence from the lowest to the highest level. Treatments were increased over time due to the possibility that the GAA might cause negative health effects on the heifers, and the increasing doses would allow the detection of any problems at the lowest amount that was problematic. In addition, the sequential increase of GAA amounts partially reduced the need for an extended adaptation

period because the heifers were adapted to the next greatest amount of GAA prior to initiating each treatment. Treatments were all provided as continuous infusions into the abomasum to preclude the potential for ruminal degradation. Treatments were infused into the abomasum through Tygon tubing (i.d. = 3.32 mm; Saint-Gobain North America, Valley Forge, PA) passed through the ruminal cannula, the reticulo-omasal orifice, and the omasum and held in the abomasum with a circular rubber flange (10 cm diameter) at one end. A peristaltic pump (Model crude protein [CP]-78002-10; Cole-Parmer Instrument Company, Vernon Hills, IL) was used to make the infusions.

The treatment solutions of GAA were prepared as 1% solutions in water; the GAA was initially solubilized with 0.22 g of 6 M HCl/g GAA (pH approximately 2.5), and then 0.11 g of 50% (wt/wt) NaOH/g GAA was added, which raised the pH to approximately 3.1. The GAA was then diluted, as necessary, so that the final weight of the solution was 4 kg/d. For those heifers receiving L-Met, it was added to the solution for each heifer and shaken until dissolved.

Heifers were housed in tie-stalls with free access to water and were limit-fed twice daily (0600 and 1800 hours) a diet (Table 1) providing (dry matter [DM)] basis) 5.3 kg/d of rolled corn, 3.6 kg/d of alfalfa, and 50 g/d of trace-mineralized salt. Using the NASEM (2016) model, basal supplies of metabolizable Met were estimated to be 17.2 g/d (assumptions: corn = 88% total digestible nutrients [TDN], 65% ruminally undegraded CP, 2.1% Met in CP; alfalfa = 60% TDN, 20% ruminally undegraded CP, 1.35% Met in CP). Because the diet contained no animal products, it was assumed devoid of GAA and creatine. Animals were observed daily for any potential symptoms of toxicity such as inappetence or depressed attitude, but no health problems were observed during the study.

Sample collection and laboratory analyses

Samples of the feed ingredients (rolled corn and alfalfa) were collected and frozen (−20 °C) on days 3 and 6 of each period for subsequent analysis. Samples were mixed within the period to obtain composite samples, dried in a 55 °C forced-air oven for 72 h to determine partial DM, ground to pass through a 1-mm screen (Thomas-Wiley Laboratory Mill Model 4, Thomas Scientific USA, Swedesboro, NJ), and stored for subsequent analysis. Feed samples were analyzed for DM, neutral detergent fiber (NDF), N, and ash. The NDF content was measured according to the technique of Van Soest et al. (1991) with α-amylase and sodium sulfite. DM content was determined by drying samples at 105 °C for 24 h in a forced-air oven. Ash was determined following combustion at 450 °C for 8 h. The N content was measured with a combustion analyzer (Nitrogen Analyzer, Leco Corporation, St. Joseph, MI), and CP was calculated as N × 6.25.

On days 3 and 6 of each period, blood samples were collected from the coccygeal vein into 10-mL heparinized blood collection tubes (Becton, Dickinson and Co., Franklin Lakes, NJ) at 4 h after the morning feeding (1000 hours). Samples were stored on crushed ice immediately after collection and then centrifuged (1,200 × g, 4 °C, 15 min) to harvest plasma. Plasma samples were frozen at −20 °C for later analyses of AA (day-6 samples only), homocysteine (day-6 samples only), GAA, creatine, and creatinine. On day 6 of each period, urine samples were collected, following stimulation of the vulva to induce urination, three times at 0700, 1000, and 0100 hours, composited, and frozen at −20 °C for later analyses of GAA, creatine, and creatinine.

Plasma free AA were determined by high-performance liquid chromatography (HPLC) after deproteinization by mixing plasma with equal volumes of 10% (wt/vol) sulfosalicylic acid

containing norleucine as an internal standard (Campbell et al., 1997). Chromatography was achieved on a Li cation-exchange column, followed by derivatization with o-phthalaldehyde and fluorescence detection (Batista et al., 2016).

Plasma homocysteine was analyzed as the carboxymethyl derivative formed as described by Tcherkas and Denisenko (2001) using cation-exchange HPLC separation with post-column o-phthalaldehyde derivatization and fluorescence detection. Samples or standards (1 mL) were placed in polyethylene tubes, and disulfide bonds were reduced with the addition of 200 μL 2-mercaptoethanol, which was mixed and allowed to set at 20 °C for 5 min. Samples were then deproteinized with 2 mL of methanol, which was mixed for 30 s. Samples were then centrifuged at 4,000 × g for 15 min at 20 °C. Supernatants (2.25 mL) were then dried in 2-mL microcentrifuge tubes under N2, then reconstituted with 50 μL water and 50 μL 2-mercaptoethanol. Carboxymethyl derivatives were produced by the addition of 200 μL of 2 M iodoacetic acid (mixed in 0.1 M sodium borate buffer adjusted to pH 11.5); sample blanks, which were subtracted from concentrations of derivatized samples, were treated with 200 μL of 0.1 M sodium borate buffer adjusted to pH 11.5. Following mixing and a 5-min reaction period, 400 μL of lithium diluent (LI220; Pickering Laboratories, Mountain View, CA) was mixed with each sample, which was subsequently centrifuged at 17,000 × g for 5 min at 20 °C and filtered through a 0.2-μm syringe filter. The HPLC analysis used a cation-exchange column (Pickering Laboratories) maintained at 33 °C, a 20-μL sample loop, and a flow rate of 0.6 mL/min. The eluant was a lithium eluant at pH 2.75 (LI275, Pickering Laboratories) for 20 min, followed by a lithium column regenerant (RG003; Pickering Laboratories) for 5 min, and re-equilibration of the column with LI275 for 20 min prior to the next sample injection. Post-column derivatization of eluted compounds with o-phthalaldehyde and 2-(dimethylamino) ethanethiol (both from

Pickering Laboratories) was followed by fluorescence detection (excitation 330 nm, emission 465 nm).

Urine and blood GAA, creatine, and creatinine were determined using HPLC according to Shingfield and Offer (1999) with some modification. Plasma samples were prepared by mixing equal volumes of 10% (wt/vol) sulfosalicylic acid and sample, vortexing, freezing overnight, centrifuging (17,000 × g, 10 min, 4 °C), and then filtering through a 0.2-μm syringe filter into HPLC vials for injection. For urine sample preparation, 100 μL of sample was diluted with 900 μL diluent, which consisted of 0.9 g of ammonium phosphate and 1.01 g of sodium 1-heptane sulfonic acid in 1 liter of deionized H_2O with pH adjusted to 2.2 with H_3PO^4. Diluted samples were filtered through a 0.2-μm syringe filter into an HPLC vial. The components of the sample were separated on a 25 cm × 4.6 mm Discovery BIO Wide Pore C18 column (5-μm particle size; Supelco Inc., Bellefonte, PA). The mobile phase consisted of 1.01 g sodium 1-heptane sulfonic acid, 0.9 g ammonium phosphate, 35 mL methanol, and 70 μL triethylamine made to 1 L with deionized H2O and adjusted to pH 2.8 with 7.5 M H_3PO^4. Compounds were detected by absorbance at 200 nm. To achieve chromatographic separation, 5 μL samples were injected to the column at 20 °C with a flow rate of 0.5 mL/min for 14 min, and then with a flow rate of 1.2 mL/min. Sample separation was completed at 25 min, and the column was flushed with 100% methanol for 10 min at 1.2 mL/min and re-equilibrated with the mobile phase for 19 min. The flow rate was then returned to 0.5 mL/min for 1 min prior to the next injection. Total run time was 55 min.

Glomerular filtration was calculated under the assumption that creatinine is entirely filtered from the blood and not reabsorbed from glomerulus. Renal reabsorption of GAA was calculated as: 1 − [(urinary GAA concentration/urinary creatinine concentration)/(plasma GAA

concentration/plasma creatinine concentration)], and renal reabsorption of creatine was calculated similarly with concentrations of creatine replacing those of GAA.

Statistical analyses

Treatments were arranged as a 5 × 2 factorial and included five amounts of GAA (0, 10, 20, 30, and 40 g/d) and two amounts of Met (0 and 12 g/d). Initial analyses were completed with plasma GAA and creatine concentrations to determine if cattle had adapted to treatments by day 3. Data from samples collected on days 3 and 6 of each period were analyzed with a repeated-measures analysis with a model including fixed effects of Met, GAA, day, and all interactions. Heifer within Met level was included as a random effect that served as the error term for testing effects of Met. Day was treated as a repeated measure with a covariance structure of compound symmetry.

Data from day 6 were analyzed with a model including fixed effects of Met, GAA, and their interaction. The GAA supplementation was considered as a repeated measure with the covariance structure of autoregressive. Heifer within Met level was included as a random effect that served as the error term for testing effects of Met. Means were separated using polynomial contrasts to test the linear and quadratic effects of GAA as well as the interactions of Met with those effects.

Results and Discussion

Creatine is synthesized, predominantly in the liver, from GAA and S-adenosylmethionine, which provides the methyl group for synthesis of creatine from GAA. Because regulation of creatine synthesis occurs at the level of GAA synthesis, the use of methyl groups in the conversion of GAA to creatine is an unregulated process (Ostojic, 2014). Thus, GAA supplementation in amounts greater than normal endogenous synthesis can induce a methyl

group deficiency and consequently hyperhomocysteinemia (Setoue et al., 2008). Supplemental methyl group sources such as Met, which acts as the methyl donor in most biological methylation, can play an important role in supplying methyl groups to produce creatine. Therefore, providing sufficient methyl groups for GAA methylation not only can increase creatine synthesis but also can prevent the methyl group deficiency which may occur as a result of GAA supplementation (Ostojic, 2014; Peters et al., 2015).

Several studies have elucidated the role of GAA as a creatine precursor for humans and as a means of improving the growth and yield of meat for broilers (Lemme et al., 2011) and growing-finishing pigs (Wang et al., 2012). In contrast, we were unaware of any research with GAA as a creatine precursor for cattle. This work was conducted as a pilot study to provide useful information about GAA utilization and to develop a GAA-induced methyl group deficiency model in cattle. Our goal was to evaluate the ability of post-ruminal supplementation of relatively large amounts of GAA to generate a methyl group deficiency in cattle and to determine.

Plasma homocysteine, GAA, creatine, and creatinine concentrations

Data (Table 2) demonstrated significant effects of day as well as interactions of day with treatment: creatine (Met × day, $P = 0.10$; GAA × day, $P = 0.08$; Met × GAA × day, $P = 0.05$), GAA (Met × day, $P = 0.07$; Met × GAA × day, $P = 0.05$). Because the plasma data demonstrated that cattle had not completely adapted to treatments by day 3 of each period, only data from day 6 were used for analyses discussed in this paper.

There was a Met × GAA-linear interaction ($P = 0.003$) for plasma homocysteine (Table 3). For heifers receiving no Met, increases in plasma homocysteine were induced by 30 and 40 g/d of GAA, whereas for heifers receiving 12 g/d of Met, there was no increase in plasma

homocysteine in response to increasing GAA supplementation. The lack of change in plasma homocysteine concentrations in response to supplemental GAA in the presence of supplemental Met could be a result of the role Met plays in providing enough methyl groups to prevent methyl group deficiency. The increases in plasma homocysteine concentrations in response to GAA infusions in the absence of supplemental Met indicated that a methyl group deficiency was generated, suggesting that consumption of methyl groups for the conversion of GAA to creatine can restrict the availability of methyl groups for other reactions in the body. The methylation of GAA also produces *S*-adenosylhomocysteine, which may contribute to elevated levels of homocysteine in blood (McBreairty et al., 2015). The elevation of plasma homocysteine can be considered a useful marker of methyl group deficiency (da Costa et al., 2005; Setoue et al., 2008). Met prevented the elevation in plasma homocysteine, likely by providing methyl groups that could aid in homocysteine remethylation or by increasing the flux of homocysteine through transsulfuration (Zhou et al., 2017).

Plasma creatine concentrations increased quadratically ($P = 0.03$) with GAA supplementation, with all amounts of supplemental GAA elevating plasma creatine except when 40 g/d of GAA was provided with no supplemental Met. It is possible that methyl group deficiency may have limited the synthesis of creatine from GAA when no Met was provided, preventing cattle from maintaining the higher rates of creatine synthesis when large amounts of GAA were provided.

In our study, supplemental GAA linearly increased ($P = 0.001$) plasma GAA concentrations from 1.08 to 2.26 mg/L, but increases in plasma GAA concentrations tended ($P = 0.06$) to be less when Met was provided, perhaps reflecting that Met produced a more effective uptake of GAA to support creatine synthesis. Some authors have reported increased

concentrations of plasma GAA in humans (Ostojic and Vojvodic-Ostojic, 2015) in response to supplementation of GAA. Also, we observed that plasma creatinine concentrations increased linearly ($P = 0.001$) with GAA administration. Because of the conversion of creatine to creatinine, it was not surprising that plasma creatinine was elevated by GAA supplementation.

Ostojic et al. (2013b) studied 20 healthy human volunteers who consumed GAA with and without methyl group donors. Total plasma homocysteine was increased significantly by oral administration of GAA alone, whereas there was no significant difference in total plasma homocysteine when GAA was supplemented along with methyl donors. Additional work was conducted by Ostojic et al. (2014b) with 48 healthy volunteers consuming three different dosages of GAA (1.2, 2.4, and 4.8 g/d) for 6 wk to determine the effects of GAA supplementation on serum and urinary metabolites. They observed plasma homocysteine increased by 1.4 (15%), 2.6 (30%), and 6.6 μM (78%) when low, medium, and high levels of GAA were provided. In addition, the authors observed that serum GAA, creatine, and creatinine increased substantially in response to GAA supplementation. These results show that increasing exogenous GAA intake can increase serum GAA and creatine. This response would be consistent with increased creatine synthesis followed by transport via the bloodstream to various tissues, such as muscle (Ostojic et al., 2014b).

McBreairty et al. (2015) studied the effects of GAA and creatine loading on tissue creatine stores for 18 to 19 d in Yucatan miniature pigs, and they found that hepatic creatine concentrations were significantly greater for creatine and GAA groups compared with the control group. In that study, GAA supplementation led to approximately 2-fold greater hepatic creatine concentrations than did creatine supplementation. Furthermore, supplementation of GAA increased muscle creatine more (~20%) than did creatine supplementation, and it significantly

increased plasma creatine concentrations (~70%) compared with the control group. Also, GAA supplementation improved tissue creatine concentration in male broiler chicks (Lemme et al., 2007; Michiels et al., 2012). Murakami et al. (2014) studied quail fed corn–soybean meal basal diets with different dietary levels of GAA (0.00%, 0.06%, 0.12%, 0.18%, and 0.24%), and they observed elevated GAA, creatine, and creatinine content of eggs with increasing dietary levels of GAA, suggesting that GAA supplementation increased GAA conversion to creatine, with increased creatine.

Urinary concentrations of GAA, creatine, and creatinine

The body needs creatine for muscle mass development. The requirement for creatine is greater for growing animals than for adults because of the need for creatine to support growing tissues and to replace creatine lost as creatinine (Brosnan et al., 2009; Brosnan and Brosnan, 2010). Because there is a limited capacity to store creatine in tissues, muscle cannot be overloaded by circulating creatine (DeGroot et al., 2018).

In our study, supplemental GAA (Table 3) quadratically elevated (initial increase followed by plateau; $P = 0.03$) urinary concentrations of GAA. The shapes of the curves tended to be affected by Met supplementation (Met × GAA-quadratic; $P = 0.06$) because heifers receiving no supplemental Met demonstrated a decrease in urinary GAA concentrations when 40 g/d GAA was supplemented, but heifers receiving 12 g/d Met did not. Considering an estimated urine output of 10 L/d, urinary GAA excretion was increased by not more than 0.4 g/d, even when the highest amount of GAA was supplemented, demonstrating that the preponderance of supplemental GAA was methylated to creatine.

Urinary concentrations of creatine increased quadratically ($P = 0.02$) in response to GAA supplementation in parallel with increases in plasma creatine. Like plasma creatine, when 40 g/d

of GAA was provided without supplemental Met, there was a decrease in urinary creatine concentrations, but this decrease was not observed when 12 g/d Met was supplemented (Met × GAA quadratic; $P = 0.03$). The increases in urinary creatine concentrations in response to GAA supplementation were associated with elevated plasma creatine concentrations, which likely resulted from increased creatine synthesis. When the body is faced with increased GAA supply, much of the GAA is methylated to creatine, but the excess creatine will be eliminated through renal excretion which is the major clearance mechanism for the body (Ostojic et al., 2014a). Similar to our results, Ostojic et al. (2014b) observed progressive increases in serum and urinary concentrations of GAA and creatine when increasing dosages of GAA (1.2, 2.4, and 4.8 g/d) were consumed by healthy humans for 6 wk.

Urinary creatinine concentration linearly decreased ($P = 0.02$) with GAA supplementation. The reason for the reduction of urinary creatinine in response to increasing amounts of GAA is unclear, but it is possible that increases in urine volume could be responsible. Increases in urine volume, however, probably could not account for the decrease in urinary creatine concentration in heifers receiving 40 g/d GAA without Met because the magnitude of drop was not as great for creatinine as for creatine.

There was a Met × GAA-linear interaction for renal reabsorption of GAA (Table 4) because renal reabsorption percentage increased with GAA supplementation when no Met was supplemented, but it decreased with GAA supplementation when 12 g/d Met was supplemented. Supplemental GAA linearly ($P < 0.01$) and quadratically ($P < 0.01$) decreased creatine reabsorption. We also observed a significant interaction between Met and GAA for creatine reabsorption (Met × GAAquadratic; $P = 0.01$) because heifers receiving 40 g/d GAA without Met demonstrated much greater reabsorption than those that received 12 g/d Met along with 40

g/d GAA. Renal reabsorption of creatine might be expected to decrease as the availability of creatine increases and the body retains less of the available creatine. Although a portion of the changes in urinary creatine concentrations in response to GAA provision can be attributed to changes in plasma concentrations, differences in renal reabsorption were more quantitatively important in affecting urinary creatine concentrations.

Heifers excreted significant amounts of creatine in the urine, even when no GAA was supplemented. Assuming urine outputs of 10 L/d, control heifers excreted about 5 g/d of creatine, which would seem to be an insensible loss of both N and methyl groups. Previous work has shown that cattle (Dinning et al., 1949), as well as other species (Joncquel-Chevalier Curt et al., 2013), excrete significant amounts of creatine, although researchers have focused more on the urinary losses of creatinine. Thus, our work, in agreement with previous observations, suggests that urinary losses of both creatine and creatinine might be of significant biological and nutritional significance, particularly with regard to requirements for methyl groups as well as for endogenous losses of N. Across our treatments, urinary concentrations of creatine ranged from 32% to 82% of creatinine. Despite statistically significant changes in urinary GAA concentrations, the amount of GAA lost in the urine was not very important quantitatively in comparison to creatinine and creatine; urinary GAA excretion ranged from 3% to 5% of creatine plus creatinine losses.

As a whole, there were notable increases in plasma and urinary concentrations of both GAA and creatine as increasing amounts of GAA were supplemented. However, two of the three heifers receiving 40 g/d of GAA without supplemental Met demonstrated marked decreases in urinary concentrations of GAA and creatine; urinary creatinine was also lower for those two heifers, but the magnitude of drop was not large. Only one of those two heifers demonstrating

low urinary GAA and creatine concentrations had a low plasma creatine concentration and neither had strikingly low plasma GAA. Although we did not observe any evidence of health problems in any of the heifers, it is possible that the unusual plasma and urinary concentrations of GAA and creatine could have been a precursor to problems that could have developed if the treatment had been extended for more than 6 d. As noted above, the treatment providing 40 g/d GAA without Met was characterized as having greater renal absorption than would have been predicted from the responses to GAA supplementation up to 30 g/d, demonstrating that the urinary concentrations of GAA and creatine could not be fully explained by changes in plasma concentrations, particularly for GAA. This suggests that some metabolic effects downstream of creatine synthesis may have been affected by the methyl group deficiency noted for that treatment. A precise explanation for the metabolic changes in those two heifers remains unclear.

Plasma amino acids

The effect of GAA and Met supplementation on plasma AA (% of total) is shown in Table 5. We report these values as percentages of total AA because GAA supplementation quadratically increased total AA concentration ($P = 0.01$; Table 5) and expression as a percentage of the total removes some of the variation associated with individual AA concentrations.

In our study, supplemental Met significantly increased plasma Met ($P < 0.001$), but GAA supplementation had no effect on plasma concentrations of Met, which suggests that the methyl group deficiency observed with the higher amounts of GAA was not severe enough to substantially disrupt Met metabolism. Because the synthesis of creatine from GAA requires a methyl group from *S*-adenosylmethionine, we hypothesized that GAA supplementation might decrease Met availability to the heifers either by increasing Met consumption for this process or

by increasing transsulfuration. The lack of effect of GAA on plasma Met concentrations might suggest that either Met consumption was not markedly increased by GAA provision or the heifers were able to compensate by remethylating homocysteine and preventing a net consumption of Met. Plasma taurine, a product of sulfur AA metabolism (Stipanuk and Ueki, 2011), tended to be greater ($P = 0.07$) when Met was supplemented. Plasma valine ($P < 0.05$) was reduced by supplementation of Met, an effect that has been attributed in Met-deficiency models to increases in protein deposition (Campbell et al., 1996); it is unknown if our heifers were Met deficient.

The synthesis of GAA occurs through the conversion of arginine and glycine to GAA (Ostojic et al., 2013a) by arginine:glycine amidinotransferase enzymatic activity in the kidney (Brosnan and Brosnan, 2004). The supply of GAA in an animal is normally limited by the renal synthesis of GAA, which is feedback regulated such that GAA production matches the need for creatine (Stead et al., 2001). This regulation prevents the wasteful production of excess creatine. Hepatic methylation of GAA, however, does not appear to be regulated, so all GAA is methylated to form creatine (McBreairty et al., 2013). A reduction in arginine:glycine amidinotransferase activity was found in rat kidney following an increase in serum concentrations of creatine, demonstrating the inhibitory effects of creatine on arginine:glycine amidinotransferase (McBreairty et al., 2013); therefore, the availability of creatine plays a role in the regulation of renal GAA production (Edison et al., 2007). Overall, GAA supplementation in amounts exceeding renal production can reduce renal GAA synthesis, can lead to an obligatory methylation reaction that would irrevocably consume methyl groups, and can have a sparing effect on arginine (Ostojic, 2015). When Dilger et al. (2013) fed young chicks arginine-deficient diets, 0.12% supplemental GAA improved growth responses compared with 0.15% creatine and

0.25% arginine supplementation, indicating that dietary arginine could be replaced by GAA for young chicks. This is consistent with our results in that plasma arginine was increased (P = 0.002) by GAA supplementation, particularly with the initial dose of GAA. Increases in plasma arginine in response to supplemental GAA are likely the result of an arginine-sparing effect of GAA supplementation, likely by inhibiting GAA synthesis in the kidney. Quadratic effects were observed for plasma ornithine (P = 0.03) and glutamate (P = 0.003) for heifers receiving GAA supplementation. The effect of GAA supplementation on plasma ornithine may relate to increases in arginine availability, which could cause more ornithine to be formed from arginine.

Plasma tryptophan, tyrosine, threonine, and asparagine linearly increased ($P \leq 0.05$) with GAA supplementation. In addition, histidine was linearly decreased (P = 0.05) and aspartic acid was linearly and quadratically decreased ($P \leq 0.01$) by supplemental GAA. The causes and consequences of these changes are unknown.

Conclusion

The results of this experiment demonstrate that post-ruminal GAA supplementation provides a way to increase creatine supply to cattle. In our study, GAA was provided through post-ruminal infusions, which precluded ruminal microbial degradation of the GAA; the potential for ruminal degradation would need to be considered if GAA were provided through the diet. For large amounts of GAA supplementation (38.5 mg/kg body weight daily), there might be a concern about a methyl group deficiency in response to GAA, although this could be ameliorated by supplemental Met. Methyl group sources other than Met may also ensure against this problem, although we did not evaluate any methyl group sources besides Met. In addition to developing a useful model to assess methyl group utilization in cattle, we were also able to demonstrate for the first time that GAA is used by cattle as a precursor for synthesizing creatine.

This research has direct implications for both the dairy and beef cattle industries, because GAA may be an economical means of improving creatine status of cattle.

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Conflict of interest statement

The authors have no conflicts of interest that may affect their ability to objectively present data in this manuscript.

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Table 2-1. Composition of experimental diet

Ingredient	% of DM
Alfalfa hay[1]	40.31
Dry-rolled corn[2]	59.13
Trace mineral salt [3]	0.56

[1] Composition (DM basis): 36% NDF, 24% CP, 11% ash.
[2] Composition (DM basis): 8% NDF, 9% CP, 2% ash.
[3] Composition (DM basis): > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.

Table 2-2. Effect of Met and GAA supplementation on plasma concentrations of GAA, creatine, and creatinine on d 3 and 6

Plasma, mg/L	Day	Met, g/d	GAA, g/d					SEM	*P*-value[1]						
			0	10	20	30	40		Day	Met	GAA	M × G	M × D	G × D	M × G × D
GAA	3	0	1.08	0.96	1.57	1.57	2.22	0.25	0.02	0.35	<0.0001	0.32	0.07	0.91	0.05
		12	0.67	1.06	1.19	2.23	2.14								
	6	0	0.98	1.36	2.08	2.63	2.59								
		12	1.19	1.26	1.37	1.85	1.94								
Creatine	3	0	27.1	30.1	32.8	37.8	35.7	2.1	<0.0001	0.51	<0.0001	0.64	0.10	0.08	0.01
		12	26.4	31.0	30.2	33.6	29.1								
	6	0	26.0	31.0	30.9	31.5	25.9								
		12	26.4	28.4	28.0	30.0	29.7								
Creatinine	3	0	9.36	9.73	11.60	10.97	10.96	0.39	<0.01	0.11	<0.0001	0.09	0.32	0.34	0.29
		12	8.49	9.85	9.74	9.89	9.88								
	6	0	9.04	9.57	10.19	9.80	9.99								
		12	8.51	8.78	9.23	9.14	9.59								

[1] M × G = Met × GAA; M × D = Met × day; G × D = GAA × day; M × G × D = Met × GAA × day.

Table 2-3. Effect of Met and GAA supplementation on plasma and urinary concentrations of homocysteine, GAA, creatine, and creatinine

		GAA, g/d						*P*-value[1]				
Item	Met, g/d	0	10	20	30	40	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
Plasma												
Homocysteine, μ*M*	0	15.4	16.4	16.1	18.5	18.2	1.0	0.27	0.32	0.38	0.003	0.43
	12	16.9	15.6	16.4	13.6	15.8						
GAA, mg/L	0	0.98	1.36	2.08	2.63	2.59	0.24	0.06	<0.001	0.62	0.03	0.26
	12	1.19	1.26	1.37	1.85	1.94						
Creatine, mg/L	0	26.0	31.0	30.9	31.5	25.9	2.4	0.83	0.42	0.03	0.46	0.07
	12	26.4	28.4	28.0	30.0	29.7						
Creatinine, mg/L	0	9.04	9.57	10.19	9.80	9.99	0.32	0.17	<0.001	0.06	0.70	0.12
	12	8.51	8.78	9.23	9.14	9.59						
Urine												
GAA, mg/L	0	67	81	116	106	65	14	0.99	0.06	0.01	0.21	0.06
	12	63	80	94	95	104						
Creatine, mg/L	0	491	618	1027	829	386	150	0.47	0.35	0.02	0.35	0.03
	12	634	666	861	782	933						
Creatinine, mg/L	0	1546	1245	1597	1174	1118	161	0.54	0.02	0.89	0.92	0.56
	12	1404	1347	1334	958	1172						

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA.

Table 2-4. Effect of Met and GAA supplementation on renal reabsorption of GAA and creatine

Metabolite	Met, g/d	GAA, g/d					SEM	*P*-value				
		0	10	20	30	40		Met	G-L	G-Q	Met × G-L	Met × G-Q
		% renal reabsorption										
GAA	0	56.8	53.6	64.7	65.9	77.7	5.6	0.05	0.47	0.11	<0.001	0.77
	12	67.2	58.7	50.2	51.1	52.1						
Creatine	0	90.2	85.2	78.0	77.7	88.6	2.5	0.10	<0.01	<0.01	0.13	0.01
	12	84.0	84.8	77.8	75.2	74.6						

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA.

Table 2-5. Effect of Met and GAA supplementation on plasma amino acid concentrations

		GAA, g/d						*P*-value[1]				
Amino acid	Met, g/d	0	10	20	30	40	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
Total amino acids, m*M*	0	2.03	2.21	2.37	1.91	2.11	0.11	0.65	0.96	0.01	0.67	0.43
	12	1.93	2.12	2.26	2.15	1.96						
		% of total amino acids										
Methionine	0	1.13	1.12	1.23	1.22	1.15	0.10	<0.001	0.15	0.36	0.40	0.98
	12	1.72	1.61	1.77	2.06	1.76						
Taurine	0	1.47	1.51	1.31	1.66	1.53	0.16	0.07	0.38	0.74	0.10	0.42
	12	1.79	2.00	1.84	1.55	1.58						
Arginine	0	5.13	5.79	5.85	5.53	6.11	0.26	0.64	0.002	0.11	0.73	0.51
	12	4.88	5.99	5.65	5.44	5.86						
Ornithine	0	3.11	3.60	3.49	2.96	3.30	0.16	0.08	0.28	0.03	0.75	0.49
	12	2.92	3.33	3.34	2.91	2.89						
Citrulline	0	3.62	3.89	3.81	3.53	3.71	0.31	0.40	0.78	0.31	0.97	0.69
	12	3.16	3.74	3.55	3.13	3.39						
Leucine	0	7.38	6.98	7.67	7.17	6.53	0.47	0.24	0.50	0.28	0.53	0.56
	12	6.76	5.89	6.42	7.33	6.02						
Isoleucine	0	6.25	5.44	6.31	5.86	5.52	0.44	0.16	0.43	0.58	0.87	0.36
	12	5.85	4.62	4.73	5.88	4.87						
Valine	0	10.93	10.85	11.41	11.58	10.89	0.60	0.05	0.70	0.69	0.96	0.50
	12	10.02	9.14	9.65	10.42	9.63						
Lysine	0	4.37	4.43	4.32	4.13	4.64	0.37	0.55	0.53	0.59	0.78	0.14
	12	4.20	4.69	4.81	4.77	4.47						
Histidine	0	3.82	3.89	3.45	3.84	3.30	0.20	0.35	0.05	0.11	0.81	0.47

	12	3.80	4.24	3.97	3.71	3.63						
Threonine	0	4.00	4.37	4.72	4.39	4.36	0.19	0.86	0.05	0.22	0.86	0.06
	12	4.26	4.12	4.28	4.52	4.50						
Tryptophan	0	1.41	1.50	1.68	1.73	1.95	0.08	0.30	<0.001	0.68	0.01	0.34
	12	1.47	1.64	1.63	1.58	1.63						
Phenylalanine	0	2.51	2.34	2.87	2.71	2.73	0.16	0.22	0.07	0.30	0.79	0.96
	12	2.83	2.65	2.89	3.23	2.84						
Tyrosine	0	2.49	2.45	3.25	2.54	3.06	0.22	0.60	0.006	0.36	0.78	0.07
	12	2.88	2.44	2.75	3.15	3.25						
Glutamate	0	3.68	3.26	3.02	3.36	3.63	0.27	0.17	0.69	0.003	0.69	0.79
	12	4.17	3.80	3.58	3.41	4.16						
Glutamine	0	15.89	14.16	13.46	14.87	15.02	1.17	0.81	0.89	0.07	0.60	0.40
	12	13.89	14.92	13.90	13.65	15.42						
Aspartic acid	0	0.62	0.56	0.47	0.49	0.52	0.05	0.39	0.001	0.01	0.55	0.99
	12	0.69	0.60	0.54	0.51	0.55						
Asparagine	0	1.68	1.73	2.04	1.66	1.84	0.10	0.95	0.001	0.98	0.03	0.87
	12	1.60	1.63	1.80	2.06	1.87						
Alanine	0	8.53	8.91	8.47	8.52	8.64	0.37	0.01	0.08	0.08	0.11	0.08
	12	11.05	9.92	9.60	9.22	9.81						
Serine	0	3.80	3.74	3.71	3.48	3.57	0.20	0.24	0.24	0.06	0.03	0.03
	12	3.81	4.03	4.08	4.26	3.82						
Glycine	0	8.00	9.37	7.39	8.63	7.92	0.48	0.92	0.17	0.14	0.58	0.57
	12	8.10	8.84	9.08	7.10	7.94						

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA.

Chapter 3 - Effects of guanidinoacetic acid supplementation on nitrogen retention and methionine flux in cattle[1]

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Abstract

Creatine stores high-energy phosphate bonds in muscle and is synthesized in the liver through methylation of guanidinoacetic acid (GAA). Supplementation of GAA may therefore increase methyl group requirements, and this may affect methyl group utilization. Our experiment evaluated the metabolic responses of growing cattle to postruminal supplementation of GAA, in a model where methionine (Met) was deficient, with and without Met supplementation. Seven ruminally cannulated Holstein steers (161 kg initial body weight [BW]) were limit-fed a soybean hull-based diet (2.7 kg/d dry matter) and received continuous abomasal infusions of an essential amino acid (AA) mixture devoid of Met to ensure that no AA besides Met limited animal performance. To provide energy without increasing the microbial protein supply, all steers received ruminal infusions of 200 g/d acetic acid, 200 g/d propionic acid, and 50 g/d butyric acid, as well as abomasal infusions of 300 g/d glucose. Treatments, provided abomasally, were arranged as a 2 × 3 factorial in a split-plot design, and included 0 or 6 g/d of L-Met and 0, 7.5, and 15 g/d of GAA. The experiment included six 10-d periods. Whole body Met flux was measured using continuous jugular infusion of 1-^{13}C-l-Met and methyl-$^{2}H_3$-L-Met. Nitrogen retention was elevated by Met supplementation ($P < 0.01$). Supplementation with GAA tended to increase N retention when it was supplemented along with Met, but not when it was supplemented without Met. Supplementing GAA linearly increased plasma concentrations of GAA and creatine ($P < 0.001$), but treatments did not affect urinary excretion of GAA, creatine, or creatinine. Supplementation with Met decreased plasma homocysteine ($P < 0.01$). Supplementation of GAA tended ($P = 0.10$) to increase plasma homocysteine when no Met was supplemented, but not when 6 g/d Met was provided. Protein synthesis and protein degradation were both increased by GAA supplementation when no Met was supplemented, but decreased by

GAA supplementation when 6 g/d Met were provided. Loss of Met through transsulfuration was increased by Met supplementation, whereas synthesis of Met from remethylation of homocysteine was decreased by Met supplementation. No differences in transmethylation, transsulfuration, or remethylation reactions were observed in response to GAA supplementation. The administration of GAA, when methyl groups are not limiting, has the potential to improve lean tissue deposition and cattle growth.

Key Words: creatine, guanidinoacetic acid, homocysteine, methionine, methionine flux

Abbreviations: AA, Amino acid; BCAA, branched-chain amino acids; BW, body weight; DM, dry matter; GAA, Guanidinoacetic acid; HPLC, high-performance liquid chromatography; PD, protein degradation; PS, protein synthesis; SAM, S-adenosylmethionine.

Introduction

Guanidinoacetic acid (GAA) can be obtained from dietary sources or mainly synthesized endogenously in the kidney from glycine and arginine (Ostojic, 2015). Creatine, because of storing energy in high-energy phosphate bonds, is known for its essential role in cellular energetics (Brosnan et al., 2009). Endogenous synthesis of creatine mainly occurs in the liver and kidney (Wyss and Kaddurah-Daouk, 2000), involving 3 amino acids (AA; arginine, glycine, and methionine [Met]) by a 2-step mechanism (Brosnan and Brosnan, 2004). The first reaction takes place mainly in the kidney, where the transfer of an amidino group from arginine to the amino group of glycine forms ornithine and GAA. GAA then enters into blood to be absorbed by the liver for the subsequent step of creatine synthesis. In the second reaction, a methyl group from S-adenosylmethionine (SAM) in an irreversible reaction is transferred to GAA to form creatine and S-adenosylhomocysteine. Creatine is then released into the blood stream to be taken up by various tissues, but mainly by muscle cells (Wyss and Kaddurah-Daouk, 2000; Riesberg et

al., 2016). Also, S-adenosylhomocysteine, resulting from the methylation reaction, is hydrolyzed to form homocysteine. Creatine synthesis is a major consumer of methyl groups in humans (Stead et al., 2006), and GAA supplementation may increase methyl group demand and affect homocysteine metabolism by increasing conversion of SAM to S-adenosylhomocysteine in the body; this can lead to increased plasma homocysteine concentrations in humans (Ostojic, 2014). Some compounds such as Met, choline, and betaine, which play key roles as methyl donors in methylation reactions in the body, may prevent GAA from causing methyl group deficiency and homocysteinemia (Tehlivets et al., 2013).

The production of GAA consumes a large amount of arginine (Brosnan et al., 2011), thus GAA supplementation is able to spare arginine by reducing GAA synthesis. Creatine as a nutritional supplement has the ability to increase muscle mass and BW in humans (Hopwood et al., 2006; Cooper et al., 2012; Gualano et al., 2012), and several studies have illustrated that dietary GAA supplementation can improve growth performance in finishing cattle (Li et al., 2020; Liu et al., 2020, 2021). The body needs creatine for permanent growth of the muscle and for replacing creatine lost by the decomposition of creatine to creatinine, which is excreted in urine (Brosnan et al., 2009; Brosnan and Brosnan, 2010).

Ardalan et al. (2020) showed that GAA supplementation to dairy heifers increased plasma and urinary creatine concentrations and created a mild methyl group deficiency (elevation of plasma homocysteine) that could be prevented with Met supplementation. In the current study, supplementation of GAA was evaluated as a means to improve nitrogen retention (growth) via conversion to creatine, but at the same time it might generate a methyl group deficiency that could have negative consequences. Thus, we evaluated GAA provision under

conditions where Met was specifically limiting and also considered how supplemental Met might affect the cattle's response to GAA.

Our hypotheses were that GAA supplementation to Met deficient cattle would induce a methyl group deficiency and that Met supplementation would reduce the methyl group deficiency. In light of those expected responses, we further hypothesized that GAA might improve N retention when Met was supplemented.

Materials and Methods

All experimental procedures involving cattle were approved by the Institutional Animal Care and Use Committee at Kansas State University.

Seven ruminally cannulated Holstein steers (161 ± 15 kg initial BW) were used in the experiment. The experiment was designed with 6 steers, and the additional steer was provided a treatment sequence identical to that of another steer. The experiment used a 2 × 3 factorial arrangement of treatments in split-plot design. Methionine supplementation amount (0 or 6 g/d) was the main-plot treatment arranged in 3 concurrent, replicated 2 × 2 Latin squares with 30-d periods; as described below, 0 and 6 g/d of Met were designed to be below and above the Met requirement of the steers. Amount of supplemental GAA (0, 7.5, or 15 g/d) was the subplot treatment, and it was provided in 10-d subplot periods within each main plot period; sequences for the GAA treatment were balanced for carryover among squares. In a previous experiment with growing Holstein heifers (520 kg BW), we observed that doses of GAA up to 40 g/d could be provided without signs of toxicity (Ardalan et al., 2020). The greatest amount of GAA used in this experiment (15 g/d) was similar to that amount on a BW basis.

Prior to the experiment, steers were adapted to the diet for 14 d. After this adaptation, steers were housed in individual metabolism crates that allowed feces and urine to be collected

separately. Steers were housed in a temperature-controlled room at 21 °C. All steers had free access to water and were limited twice daily (0700 and 1900 hours).

The experimental approach was based on the Met-deficient model described by Campbell et al. (1997). All steers were maintained under conditions of a Met deficiency through a diet designed to provide deficient amounts of Met (Campbell et al., 1997); the diet principally contained soybean hulls, wheat straw, and molasses (Table 1) and was offered in amounts of 2.7 kg dry matter (DM)/d. The basal diet (Campbell et al., 1997) was formulated to contain only small amounts of ruminally undegraded protein and therefore provided a low metabolizable protein:energy ratio. The diet was designed to provide adequate ruminally degraded protein through ruminal infusion of 10 g urea/d to support maximal microbial growth (Campbell et al., 1997), assuring that changes in nitrogen recycling, which could be affected by treatments, did not affect ruminal microbial growth. To prevent limitations by AA other than Met, all steers received continuous abomasal infusions of a supplemental AA mixture containing deficient amounts of Met. Also, to provide energy and prevent it from being limiting, without increasing microbial protein supply, additional energy was supplied to steers through continuous ruminal infusions of volatile fatty acids and abomasal infusions of 300 g/d glucose. Ruminal infusates for each steer provided 200 g/d acetic acid, 200 g/d propionic acid, and 50 g/d butyric acid as energy sources, and 10 g/d of urea as a source of ruminally available N, with water added to bring the final weight of the mixture to 4 kg/d. The ruminal solution was infused through Tygon tubing (i.d. = 3.32 mm; Saint-Gobain North America, Valley Forge, PA) passed through the ruminal cannula and held in the rumen with a perforated vial attached to the end of the ruminal infusion lines. A peristaltic pump (Model CP-78002-–10; Cole-Parmer Instrument Company, Vernon Hills, IL) was used to make the ruminal and abomasal infusions.

The basal abomasal infusate containing AA was prepared daily as follows. All steers received a basal infusion of a mixture providing daily 20 g of L-leucine, 15 g of L-isoleucine, 15 g of L-valine, 2 g of L-Met, 20 g of L-lysine-HCl, 15 g of L-threonine, 8 g of L-histidine-HCl-H2O, 20 g of L-phenylalanine, 5 g of L-tryptophan, 15 g of L-arginine, and 150 g of monosodium glutamate. The branched-chain AA (BCAA: L-valine, L-isoleucine, and L-leucine) was initially solubilized in ~1 kg of water containing 60 g of 6 M HCl. Once BCAA dissolved, the remaining AA, except sodium glutamate, were added and mixed until dissolved. After all AA were dissolved, the sodium glutamate was mixed with the basal AA mixtures and water was added to bring the final weight of the daily infusate to 2 kg.

For preparing the GAA treatments, a 1% (wt/wt) solution of GAA was prepared in water; the GAA was initially solubilized with 0.22 g of 6 M HCl/g GAA, and then 0.11 g of 50% (wt/wt) NaOH/g GAA was added.

All steers received daily supplementation with 10 mg/d folic acid, 0.10 mg/d cyanocobalamin (B_{12}), and 10 mg/d pyridoxine (B_6) to ensure that the steers were not deficient in these vitamins (Lambert et al., 2004). These vitamins were mixed with the abomasal infusate. To prepare the vitamin supplements, 1 g folate was dissolved into 200 mL 50% (wt/wt) acetic acid, and 0.01 g cyanocobalamin and 1 g pyridoxine were dissolved in 200 mL water; 2 mL of each solution were added to each steer's infusate daily.

Abomasal infusates for each steer were prepared by mixing 2 kg of the basal AA solution, 330 g of dextrose, the amount of GAA solution required to provide the treatment amount of GAA, and 2 mL of each of the 2 vitamin mixtures. Dry L-Met was added to the abomasal infusate based on treatment and allowed to dissolve. Water was then added to bring the total weight of the daily infusate to 4 kg. The abomasal infusate was provided continuously into

the abomasum through Tygon tubing (i.d. = 3.32 mm; Saint-Gobain North America) passed through the ruminal cannula, the reticulo-omasal orifice, and the omasum and held in the abomasum with a circular rubber flange (10 cm diameter).

The diet was estimated to provide 2.7 g/d metabolizable Met to the steers, based on measurement of nutrient flows from cattle fed a similar diet (Campbell et al., 1997). Thus, basal supplies of metabolizable Met to all steers were 4.7 g/d (2.7 g/d from the diet plus 2 g/d in the basal abomasal infusions). Two grams per day of Met in the basal infusate were included to prevent the Met deficiency from being so extreme that the steers would be at risk of metabolic derangement. For similar steers, the Met requirement for maximal N retention was 7.9 g/d (Campbell et al., 1997), so the steers receiving no supplemental Met were predicted to be deficient in Met supply, whereas, for steers receiving 6 g/d supplemental Met and having metabolizable Met supplies of 10.7 g/d, the requirement was predicted to be exceeded.

Sample collection and laboratory analyses

Feed analyses. Feed samples were collected from days 4 through 9 of each period and mixed within period to obtain composite samples. Feed samples were dried in a forced-air oven for 72 hr (55 °C) and then ground to pass through a 1-mm screen (Thomas-Wiley Laboratory Mill Model 4, Thomas Scientific, Swedesboro, NJ) and stored for subsequent analysis. Feed refusals, if any, were collected from days 5 through 10 of each period, composited by period, dried at 55 °C in a forced-air oven for 72 hr, and ground to pass a 1-mm screen for later analysis. Total DM of feed and orts were determined by drying samples at 105 °C for 24 hr in a forced-air oven, and ash was determined by combustion at 450 °C for 8 hr.

Blood sampling and analysis. On days 6, 8, and 10 of each period, blood samples were collected from a jugular vein into 10-mL heparinized blood collection tubes (Becton, Dickinson

and Co., Franklin Lakes, NJ) at 2 hr after the morning feeding (0900 hours). Samples were stored on crushed ice immediately after collection and then centrifuged (1,000 × g, 4 °C, 15 min) to harvest plasma. Plasma samples were frozen at −20 °C for later analyses of AA, GAA, creatine, and creatinine. Plasma AA (day 10 samples only) were measured by gas chromatography using a commercial kit (EZ:faast; Phenomenex, Torrance, CA) with the exception of homocysteine and cysteine (days 6, 8, and 10 of each period), which were measured by HPLC. Plasma homocysteine and cysteine concentrations were analyzed as 4-fluoro-7-sulfobenzofurazan adducts by reversephase HPLC as described by Pfeiffer et al. (1999). Urine and blood GAA, creatine, and creatinine were determined using HPLC according to Shingfield and Offer (1999) with some modification. Plasma samples were prepared by mixing equal volumes of 10% (wt/vol) sulfosalicylic acid and sample, vortexing, freezing overnight, centrifuging (17,000 × g, 10 min, 4 °C), and then filtering through a 0.2-μm syringe filter into HPLC vials for injection. For urine sample preparation, 100 μL of sample was diluted with 900 μL diluent, which consisted of 0.9 g of ammonium phosphate and 1.01 g of sodium 1-heptane sulfonic acid in 1 L of deionized H_2O with pH adjusted to 2.2 with H_3PO_4. Diluted samples were filtered through a 0.2-μm syringe filter into an HPLC vial. The components of the sample were separated on a 25 cm × 4.6 mm Discovery BIO Wide Pore C18 column (5-μm particle size; Supelco, Bellefonte, PA). The mobile phase consisted of 1.01 g sodium 1-heptane sulfonic, 0.9 g ammonium phosphate, 35 mL methanol, and 70 μL triethylamine made to 1 L with deionized H_2O and adjusted to pH 2.8 with 7.5 M H_3PO_4. Compounds were detected by absorbance at 200 nm. To achieve a chromatographic separation, 5 μL sample was injected to the column at 20 °C with a flow rate of 0.5 mL/min for 14 min, then with a flow rate of 1.2 mL/min. Sample separation was completed at 25 min, and the column was flushed with 100% methanol for 10 min at 1.2 mL/min

and re-equilibrated with the mobile phase for 19 min. The flow rate was then returned to 0.5 mL/min for 1 min prior to the next injection. Total run time was 55 min.

Nitrogen retention. On days 5 through 10 of each period, total feces and urine for each steer were collected and weighed daily to quantify output. The urinary output was collected into buckets containing 900 mL of 10% (wt/wt) H_2SO_4 to maintain pH below 3 for preventing ammonia loss and limiting microbial growth. Urine and feces were collected, weighed, and sampled daily; 1% of urine and 10% of feces were saved and frozen (–20 °C) for subsequent analysis.

Nitrogen retention was measured over three 2-d collection periods (i.e., days 5 and 6, 7 and 8, and 9 and 10). The total nitrogen content of diet, orts, wet feces, and urine was determined using a combustion analyzer (True Mac; Leco Corporation, St. Joseph, MI). Nitrogen retention was determined as the difference between N intake (feed + infusates − refusals) and N excretion in feces and urine.

Glomerular filtration was calculated with the assumption that creatinine is entirely filtered from the blood and not reabsorbed from glomerulus. Thus, renal reabsorption of GAA was calculated as (1 − ((urinary GAA concentration/urinary creatinine concentration)/(plasma GAA concentration/plasma creatinine concentration))), and renal reabsorption of creatine was calculated similarly with concentrations of creatine replacing those of GAA (Verouti et al., 2021). These renal reabsorptions were calculated for 2-d windows, matching the day 6 plasma concentrations with the urinary concentrations. from days 5 and 6, the day 8 plasma concentrations with the urinary concentrations from days 7 and 8, and the day 10 plasma concentrations with the urinary concentrations from days 9 and 10.

Methionine flux measurement. On day 10 of each period, whole body Met flux was measured by continuously intravenously infusing two labeled Met sources (1-^{13}C-L-Met [99%] and methyl-$^{2}H_3$-L-Met [98%], both from Cambridge Isotope Laboratories, Inc., Tewksbury, MA). The labeled Met (0.04 g/hr of each label), following a pulse dose of 0.04 g of each label, were infused through a jugular catheter (MILACATH #LA1420; MILA International, Inc., Florence, KY) that was placed earlier in the day. The infusion period started 4 hr after feeding and lasted 4 hr (Preynat et al., 2009). Blood samples for measuring background enrichment were collected 2 hr prior to initiation of label infusion (i.e., 2 hr after morning feeding) and enriched samples were collected at the end of the 4-hr infusion period into 10-mL heparinized blood collection tubes (Becton, Dickinson and Co., Franklin Lakes, NJ). Samples were stored on crushed ice immediately after collection and then centrifuged (1,000 × g, 4 °C, 15 min) to harvest plasma. Plasma samples were stored at −20 °C for measuring enrichment of label. Enrichments of the 1-^{13}C-L-Met and methyl-$^{2}H_3$-L-Met in plasma were determined using gas-liquid chromatography/mass spectrometry analysis according to Loest et al. (2002).

Methionine flux was calculated according to the procedure of Preynat et al. (2009). The whole body flux of methyl-$^{2}H_3$-l-Met was defined as: Q(methyl-$^{2}H_3$-L-Met) = protein synthesis (PS) + transmethylation reactions = protein degradation (PD) + dietary intake + remethylation. The whole body flux rate of 1-^{13}C-L-Met was defined as: Q(1-^{13}C-L-Met) = PS + transsulfuration = PD + dietary intake. Protein synthesis was calculated as the sum of PD and Met deposition. Methionine deposition was predicted as: N retention, g N/hr × 6.25 g protein/g N × 0.134 mmol Met/g protein (Ainslie et al., 1993). Metabolizable Met intake was calculated as abomasally infused Met plus metabolizable Met provided by the diet (6.71 mmol metabolizable Met/kg DM intake; Campbell et al., 1997).

Statistical analyses

Data were analyzed as a split-plot design using the Mixed procedure of SAS System 9.3 for Windows (SAS Inst. Inc., Cary, NC). For data without repeated measures, fixed effects in the model included main-plot period, Met treatment, subplot period within main-plot period, GAA treatment, and Met × GAA. Random effects included steer and steer × main-plot period (as the main-plot error term). For data with repeated measures within a sub-plot period, day and all interactions of day with treatment were included as fixed effects, and day was considered as a repeated measure with the covariance structure of autoregressive (1). Means were separated using polynomial contrasts to test the linear and quadratic effects of GAA as well as the interactions of Met with those effects. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Data for 1 steer were removed from periods 2 and 3 due to low feed intake on sampling days. Methionine flux measures were not available for one period due to failure of the pump infusing the label.

Results and Discussion

In our previous research with postruminal supplementation of GAA to cattle (Ardalan et al., 2020), we found evidence of treatment interactions with time for plasma concentration of GAA, creatine, and creatinine, suggesting that cattle had not adapted completely to treatment by day 3 (52 hr after treatment initiation). In the current study with a longer period before the first sampling time, there were no interactions between treatment and time for retention or urinary excretion of nitrogen or for plasma concentrations or renal reabsorptions of GAA, creatine, or creatinine; thus, the presented data for those measures represent all sampling times for those measure (i.e., days 5 through 10 for nitrogen retention and urinary excretion data; days 6, 8, and 10 for plasma concentrations). There was, however, an interaction between treatment and time

for plasma homocysteine (data not shown), suggesting that some minor adaptation in plasma metabolites was occurring after day 6. Therefore, only the plasma homocysteine and cysteine data from blood collected on day 10, which would allow the longest adaptation to treatments, are presented.

Dry matter intake, DM and organic matter digestibilities, and N retention

Dry matter intakes were similar among treatments as expected with limit feeding. DM and organic matter digestibilities were increased ($P < 0.01$) with the administration of Met (Table 2). The greater DM and organic matter digestibilities correspond with less fecal N excretion when Met was supplemented ($P < 0.01$). In general, we have not observed changes in organic matter digestibility when steers maintained in a similar model (Loest et al., 2002; Awawdeh et al., 2004) were supplemented with Met, and the mechanism whereby Met supplementation increased DM and organic matter digestibilities has no clear explanation. No differences were detected for DM and organic matter digestibilities in response to supplementation of GAA. Opposite of our results, Li et al. (2020), who evaluated effects of increasing levels of GAA (0, 0.3, 0.6, and 0.9 g GAA/kg DM) fed to Angus bulls, indicated that DM intake and ruminal volatile fatty acid concentrations increased when 0.6 or 0.9 g GAA/kg DM was fed. Additionally, feeding 0.9 g GAA/kg DM increased digestibilities of DM and organic matter. The authors suggested that GAA might improve DM intake by increasing nutrient digestibility, ruminal volatile fatty acid concentration, and blood creatine concentration (Li et al., 2020), which can stimulate food intake (Galbraith et al., 2006). Because we infused GAA to the abomasum, it would be unlikely to stimulate ruminal fermentation in our experiment, which could explain our lack of response to GAA for digestibility.

By design, the GAA treatments led to increases in total N intake (Table 2). Urinary (P = 0.01) and fecal ($P < 0.01$) N excretion decreased when Met was supplemented. The observed reductions in urinary and fecal N resulted in increased ($P < 0.01$) N retention from 26.4 to 32.7 g/d with Met supplementation, which was expected because Met was designed to be the most limiting AA in our experimental model (Campbell et al., 1997).

A linear (P = 0.001) increase in urinary N and a smaller quadratic (P = 0.02) increase (greatest for the intermediate GAA amount) in fecal N were observed when GAA was supplemented. Nitrogen retention responses to GAA were dependent on the Met status of the steers (Met × GAA-linear, P = 0.10). For steers receiving 6 g/d of Met, there tended to be an increase in N retention in response to increasing GAA supplementation, whereas for steers not receiving supplemental Met there was no response. The lack of a N retention response to 7.5 g/d of GAA in the presence of supplemental Met might have been due to an off-setting decrease in endogenous production of GAA; based on basal urinary excretion of GAA (0.52 g/d), creatine (5.7 g/d), and creatinine (5.7 g/d), endogenous production of GAA should be near 11.5 g/d. Indeed, the 7.5 g/d amount of GAA, because of feedback inhibition of endogenous GAA synthesis (Wyss and Kaddurah-Daouk, 2000), may not have markedly changed total creatine production or methyl group utilization for creatine synthesis. However, at the supplementation level greater than endogenous production (i.e., 15 g/d), the increase in total GAA/creatine availability seemed to improve animal production. In contrast, supplemental GAA had only a nonsignificant, slightly negative effect on nitrogen retention in steers receiving no supplemental Met, perhaps reflecting protein deposition might not be strongly affected by deficiency of methyl-group donors. Taken as a whole, these results support the concept that under conditions of methyl group deficiency, increases in creatine production may not improve nitrogen retention

(cattle growth). These observations provide a starting point for further research on the effects of GAA supplementation on increasing lean tissue deposition in growing cattle.

Li et al. (2020) fed Angus bulls a diet based on corn silage and corn grain with dietary GAA at levels of 0, 0.3, 0.6 and 0.9 g/kg DM. Daily gain, BW, and feed conversion improved when GAA was supplemented with the maximal responses to GAA achieved with 0.6 or 0.9 g GAA/kg DM. The steers receiving 0.6 g GAA/kg DM would have consumed about 6 g/d of GAA, with an expectation that only about half of that amount would be available to the steers (Speer et al., 2020); thus, the optimal amount of GAA for steers in the study of Li et al. (2020) was considerably less than in our experiment, and the difference would be even greater if expressed on a BW basis.

In a study conducted by Lemme et al. (2010), broilers received 0.8 g GAA/kg feed or 1.0 g creatine/kg feed in a Met-deficient plant-based diet (i.e., low dietary creatine concentrations) or the same diet supplemented with Met (0.37% vs. 0.57%). For broilers fed Met-adequate diets, GAA and creatine improved weight gain, breast meat yield, and feed intake, whereas GAA and creatine supplementation were ineffective for broilers fed the Met-deficient diet. This demonstrates that GAA and creatine supplements can be more effective when Met is not limiting.

Lemme et al. (2007a) fed male broilers 4 dietary GAA concentrations (0, 0.20, 0.40, or 0.60 g GAA/kg) in a vegetable based diet. Increasing GAA supplementation increased muscle creatine by about 14%, suggesting that GAA can act as a source of creatine. Additional work by Lemme et al. (2007b) compared the administration of different dietary levels of GAA (0, 0.6, 1.2, 1.8, and 2.4 g GAA/kg) when plant-based diets containing 6% meat and bone meal were fed to broilers. These researchers reported that 0.6 g GAA/kg improved gain:feed and BW gain of

female broilers. Also, supplementation with 0.6 g GAA/kg and 1.2 g GAA/kg enhanced breast meat yield in male and female broilers, respectively. These results suggest that dietary supplementation of GAA can improve broiler performance, and the optimal supplementation level of GAA is between 0.6 g GAA/kg and 1.2 g GAA/kg, with an average of 0.7 g GAA/kg diet.

Plasma and urinary concentrations of GAA, creatine, and creatinine

There were no significant interactions between GAA and Met for plasma GAA, creatine, and creatinine. Supplemental GAA linearly ($P < 0.01$) and quadratically (both 7.5 and 15 g/d GAA led to similar increases; $P = 0.02$) increased plasma GAA concentrations from 0.45 to 0.62 mg/L (Table 3). These results are in agreement with our previous research conducted on GAA supplementation to dairy heifers (Ardalan et al., 2020). Ardalan et al. (2020) showed that plasma GAA concentrations increased with increasing levels of GAA supplementation from 0.98 to 2.59 mg/L, although the rise in plasma GAA tended to be less when cattle were provided Met. These responses support the conclusion that GAA was absorbed by the gut and made available to the body. These results are also consistent with other reports of GAA administration in humans (Ostojic and Vojvodic-Ostojic, 2015), where GAA administration led to increases in plasma concentrations of GAA.

Plasma creatine concentrations increased linearly ($P < 0.01$) with increasing levels of GAA supplementation (Table 3). A reduction in plasma creatine concentrations was observed in response to Met supplementation ($P < 0.01$), suggesting that the greater tissue growth in response to Met might enhance creatine uptake into muscle. Our data are consistent with our previous research (Ardalan et al., 2020) on dairy heifers that demonstrated that plasma creatine concentrations increased at levels up to 30 g/d of supplemental GAA. These data support a

conclusion that creatine synthesis was increased in cattle through GAA supplementation. Increases in plasma creatine concentrations in response to GAA supplementation are also consistent with the work of Li et al. (2020), where feeding GAA to bulls increased blood creatine concentrations, suggesting that GAA was utilized for creatine synthesis (Li et al., 2020). There were no treatment effects on plasma creatinine concentrations ($P \geq 0.31$), in agreement with research conducted on GAA supplementation to pigs (He et al., 2018) and humans (Ostojic et al., 2013b).

No significant differences among treatments were observed for urinary excretion of GAA, creatine, or creatinine (Table 3). There was, however, a tendency (Met × GAA-quadratic; $P = 0.07$) for the intermediate level of GAA to increase urinary creatine when no Met was supplemented but to decrease it when 6 g/d Met was provided; a similar pattern was observed for urinary GAA excretion ($P = 0.11$). Ostojic et al. (2013b) orally supplemented GAA (2.4 g/d) in combination with and without betaine (methyl group donor) to healthy humans and observed increases in serum and urinary creatine when GAA was supplemented with or without supplemental betaine. Supplementation of GAA without betaine increased serum and urinary GAA, but feeding GAA with betaine increased serum GAA but had no effect on urinary excretion of GAA. Thus, when methyl donors are inadequate to support additional methylation of GAA to creatine, GAA is removed via urine. Although statistically weak, our responses for urinary GAA suggest that a similar mechanism may be in place for cattle when Met is provided as the methyl donor.

Both GAA and creatine showed linear increases (Table 3) in renal reabsorption in response to supplemental GAA. However, it is interesting to note that the creatine and GAA reabsorption increased when the plasma concentration of GAA and creatine had also raised

following GAA administration. These results are opposite our observations for creatine reabsorption in our previous work (Ardalan et al., 2020), where, in response to GAA supplementation, urine concentration of creatine increased relatively more than did plasma concentration. There are several possible explanations for increasing renal reabsorption of creatine. Both a specific plasma membrane Na+/Cl− transporter and a creatine transporter have important roles in tissue uptake and reabsorption of creatine (Jonquel-Chevalier Curt et al., 2015). The creatine transporter is saturable (Walzel et al., 2002), and *K*m value for bovine creatine transporter has been determined as 188 μM creatine (25 mg/L; Dodd et al., 1999), although studies evaluating the *K*m for creatine transporter have shown varied *K*m for different animal species. Therefore, the amount of creatine transporter and the *K*m value are important factors in regulating intracellular creatine levels (Dodd et al., 1999). Our plasma creatine concentrations were slightly less than those in our previous work (Ardalan et al., 2020), and near the *K*m of the bovine creatine transporter. Therefore, it is possible that some of the discrepancy between the current experiment and previous work is because the greater blood creatine concentrations by Ardalan et al. (2020) led to shifting the clearance of creatine from the muscle to renal excretion.

Creatine and GAA clearance in the body can be affected by others factors such as (1) creatine and GAA competition for reabsorption by the renal tubules (Sims and Seldin, 1949), (2) age (Almeida et al., 2004; Olah et al., 2019), (3) differences among species in glomerular filtration rate (Skotnickaet al., 2007), (4) effect of AA on glomerular filtration rate (Grossman, 1945; Lee and Summerill, 1982), and (5) sex hormones and gender differences (Joncquel-Chevalier Curt et al., 2013). Any of these factors, except species, could have contributed to

differences between our previous work and the current experiment in urinary creatine excretion responses as GAA was supplemented.

The body removes excess GAA that does not become methylated to creatine via renal excretion to counteract increases in GAA concentrations (Ostojic et al., 2013b). The very low amounts of GAA in urine demonstrated that most of the supplemental GAA was used for creatine synthesis. Ardalan et al. (2020) observed increases in urinary concentrations of GAA and creatine when up to 30 g/d GAA was supplemented, but the increases in urinary concentrations of GAA were small compared with the increases in urinary creatine. These data demonstrate the effectiveness of GAA for creatine formation.

Tossenberger et al. (2016) observed that broiler chicks fed 0.6 g GAA/kg feed (38 mg/kg $BW^{0.75}$ daily) or 6.0 g GAA/kg feed (359 mg/$kg^{0.75}$ daily) for 35 d, demonstrated increases in urinary excretion of GAA, creatine, and creatinine. Increases in urinary excretion of GAA, creatine, and creatinine by birds fed 0.6 g GAA/kg feed represented 16.4%, 2.5%, and 3.8%, respectively, of the supplemental GAA, whereas for birds fed 6.0 g GAA/kg feed the urinary losses of GAA, creatine, and creatinine represented 27.6%, 7.1%, and 18.3% of the supplemental GAA. These data demonstrate that renal excretion is a route of elimination of excess GAA and its metabolites creatine and creatinine in chicks.

Methionine flux

The body's Met pool, which is derived from dietary sources, PD, and de novo synthesis (homocysteine remethylation), can be used for PS or transmethylation reactions. In our experiment, the methyl-labeled Met would be removed from the Met pool through PS or in transmethylation reactions. In contrast, 1-^{13}C-L-Met is only removed from the Met pool through PS and transsulfuration reactions, because the 1-^{13}C-label is not removed during

transmethylation. Subsequent to transmethylation, removal of the 1-^{13}C-label does not occur if the labeled homocysteine is remethylated to Met, but the label is lost from the Met pool (contributing to the flux of 1-^{13}C-Met) during transsulfuration. This differential partitioning of the 2 labeled Met molecules allows estimation of Met movement through reactions of transmethylation, transsulfuration, and remethylation of homocysteine.

The effects of GAA and Met supplementation on Met flux and related measures are shown in Table 4. Methionine yielded a number of significant main-effect responses, whereas GAA yielded no main-effect responses. There were some significant interactions between the GAA and Met treatments, but the larger magnitude responses to Met are discussed first, followed by a discussion of the interactions between GAA and Met.

Effects of methionine

Supplemental Met increased ($P < 0.01$) 1-^{13}C-L-Met flux (irreversible loss rate of the Met skeleton). A sizeable part of the 2.90 mmol/hr increase in Met flux can be accounted by the 1.68 mmol/hr of Met supplemented. In contrast, the methyl-$^{2}H_3$- L-Met flux was not affected by supplementation of Met ($P = 0.17$).

Methionine supplementation increased metabolizable Met supply ($P < 0.01$; by experimental design) and Met deposition ($P < 0.01$; estimated from N retention). Protein synthesis tended to increase ($P = 0.10$) in response to Met supplementation, but PD was not significantly affected by Met supplementation. Because Met deposition was increased by Met supplementation, the difference between PS and PD was obligatorily increased.

Transmethylation reactions (i.e., homocysteine production) were not affected by Met supplementation, which is an interesting observation that suggests that transmethylation reactions may not have been limited by Met availability. Transsulfuration reactions were

increased ($P < 0.0001$) with Met supplementation, whereas remethylation reactions decreased ($P = 0.02$) when Met was supplemented. The increase in transsulfuration reaction and reduction in remethylation reaction matches reasonably well with decreases in plasma serine and increases in plasma cysteine when supplemental Met was provided. Serine is required for the conversion of homocysteine to cysteine and thus it is frequently observed that Met supplementation to Met-deficient cattle leads to decreases in plasma serine (Titgemeyer and Merchen, 1990; Lambert et al., 2002). Elevated hepatic SAM inhibits activity of enzymes that catalyze homocysteine remethylation (Mato et al., 2008). Consequently, Met supplementation, which leads to elevated hepatic SAM content and to the inhibition of remethylation reactions, can shift the metabolism of homocysteine away from remethylation and toward transsulfuration.

Interactions between GAA and methionine

There were no main effects of GAA on Met flux and related measures in Table 4, but there were interactions between GAA and Met, suggesting that the Met status of the cattle affected their response to the supplemental GAA. The whole-body flux of methyl-$^{2}H_3$-L-Met increased in response to GAA provision when GAA was provided along with no supplemental Met, but, when steers received GAA along with 6 g/d Met, the flux of Met methyl groups was decreased by GAA provision (Table 4; Met × GAA linear, $P = 0.03$). A Met × GAA-linear interaction ($P = 0.04$) was observed for flux of 1-^{13}C-L -Met, which was increased by GAA supplementation when steers received no Met, but decreased by GAA supplementation when the steers received 6 g/d Met.

There were interactions between Met and GAA for both PS and PD (Met × GAA-linear; $P = 0.04$), where both measures followed a pattern similar to those observed for 1-^{13}C-L-Met and methyl-$^{2}H_3$-L-Met. Specifically, both PS and PD increased in response to GAA supplementation

when Met was not supplemented, but decreased in response to GAA supplementation when 6 g/d Met was supplemented. Because PS and PD followed a similar pattern in response to treatments, the net effect was that protein deposition yielded a response different than that for PS. Deposition of Met was not statistically affected by GAA supplementation. The lack of an interaction between Met and GAA for Met deposition is in contrast to the observation discussed above that N retention tended ($P = 0.10$) to increase in response to supplemental GAA when Met supply was adequate. Although the pattern for Met deposition was similar to that for N retention, the lack of any statistical effect for Met deposition may be attributed to observations that were missing from the flux measurements, which reduced statistical power.

In our experiment, GAA supplementation had no significant effect on methylation reactions (homocysteine synthesis), transsulfuration, or remethylation, and no interactions between GAA and Met for methylation, transsulfuration, and remethylation reactions were detected. If all of the supplemental GAA (up to 5.3 mmol/hr) were methylated to creatine, the 1.68 mmol/hr (6 g/d) of supplemental Met would be inadequate for completely methylating the GAA. Moreover, our results showed that total methylation reactions were not increased by supplemental GAA. Thus, if the supplemental GAA was methylated, then the rate of other transmethylation reactions, such as choline synthesis, would have to correspondingly decrease for total methylation reactions to be unaffected by the GAA supplementation. In agreement with this suggestion, McBreairty et al. (2013, 2015), working with Yucatan miniature pigs receiving GAA through venous infusion or diet, demonstrated the increased methyl demand created by GAA use for creatine synthesis led to reductions in phosphatidylcholine synthesis.

Plasma AA

The effect of GAA and Met supplementation on plasma AA is shown in Table 5. As expected, Met supplementation increased plasma Met concentrations ($P < 0.001$), but plasma Met was not affected by GAA treatment. Our response in plasma Met concentrations agrees with our previous work (Ardalan et al., 2020) which suggested that methyl group deficiency induced by administration of GAA was not severe enough to alter plasma Met concentrations. Li et al. (2020) and Liu et al. (2020, 2021) also observed no effect of GAA administration to Angus bulls on plasma Met concentrations.

Plasma concentrations of homocysteine were reduced ($P < 0.01$) in response to supplemental Met. Supplementation of GAA tended to increase plasma homocysteine in steers receiving no Met quadratically (the intermediate amount of GAA increased homocysteine the most), but GAA did not increase plasma homocysteine in steers receiving supplemental Met (Met × GAA-quadratic; $P = 0.10$). The lack of an increase of plasma homocysteine concentrations in response to supplemental GAA in the presence of supplemental Met could show a role of Met in providing enough methyl groups to prevent a methyl group deficiency. Therefore, administration of Met, as a methyl donor, reduced homocysteine levels (Zhou et al., 2016). These results agree with our previous research (Ardalan et al., 2020) conducted on GAA supplementation to dairy heifers; heifers receiving 12 g/d of Met demonstrated no increase in plasma homocysteine in response to GAA supplementation. In contrast, the heifers receiving no supplemental Met showed elevated concentrations of plasma homocysteine when either 30 or 40 g/d of GAA was provided. In our previous research (Ardalan et al., 2020), plasma homocysteine was increased 20% with GAA supplementation up to 30 g/d (0.06 g GAA/kg BW daily) when no Met was provided, whereas in the current experiment an 8% increase in plasma homocysteine

concentrations was observed when 15 g/d of GAA (0.09 g GAA/kg BW daily) was supplemented without Met. The increases in plasma homocysteine concentrations in response to GAA infusions with no supplemental Met would suggest that a methyl group deficiency was generated by increasing the consumption of methyl groups for creatine synthesis from GAA. As noted previously, this could result in reduced availability of methyl groups for transmethylation reactions other than creatine production (e.g., choline synthesis, McBreairty et al., 2015).

In studies conducted on Angus bulls receiving GAA, Li et al. (2020) and Liu et al. (2020, 2021) demonstrated no differences in blood homocysteine when GAA was supplemented. We speculate that this lack of change in blood homocysteine reflects that the cattle were not deficient in Met supply, which agrees with our responses when 6 g/d of Met was supplemented. The lack of change in plasma homocysteine in those studies also could be the result of the relatively low amount of GAA that was fed (0.6 g GAA/kg diet DM).

Plasma homocysteine is considered a good marker for methyl group deficiency (da Costa et al., 2005; Setoue et al., 2008). Work with rats (Stead et al., 2001; Fukada et al., 2006) and humans (Ostojic et al., 2014, 2013a) demonstrated increases in plasma homocysteine when GAA was supplemented, and Stead et al. (2001) showed that rat hepatocytes treated with GAA had increased export of homocysteine. Thus, elevation of plasma homocysteine concentration after treatment with GAA can be linked to the increased demand for methyl groups to convert GAA to creatine (Fukada et al., 2006). In rodents, the hyperhomocysteinemia induced by GAA can be suppressed by supplementation with choline or betaine as sources of methyl groups (Setoue et al., 2008). Hence, supplemental methyl group sources such as Met can play an important role in preventing homocysteine accumulation by increasing homocysteine remethylation or by increasing the flux of homocysteine through transsulfuration (Zhou et al., 2016). Data from our

flux measurements (Table 4) demonstrated that transsulfuration was increased by Met supplementation, whereas homocysteine remethylation was reduced; these changes reflect the role of Met not only as a methyl donor but also as an important regulator of Met-regulating reactions (Finkelstein and Martin, 1986).

Plasma cysteine concentrations were increased by Met supplementation ($P < 0.01$). This observation is consistent with the greater fluxes of Met through transsulfuration when Met was supplemented (Table 4). Under normal physiological conditions, the balance between the homocysteine production and elimination maintains the homocysteine balance in the body. An animal's methyl group status is a critical determinant of the partitioning of homocysteine between transsulfuration (irreversible degradation to cysteine) and remethylation (recycling to Met) pathways. Overall, when dietary intake of Met is sufficient and Met does not need to be conserved, excessive intake of Met leads to decreased remethylation of homocysteine and increased transsulfuration of homocysteine (Nygard et al., 1999); lower availability of Met diverts homocysteine away from transsulfuration and consequently increases remethylation (Blom and Smulders, 2011).

Plasma cysteine concentrations increased quadratically ($P = 0.06$) with GAA supplementation with the greatest increase resulting from the intermediate amount of GAA, although differences were not particularly large. Although there was no effect of GAA on transsulfuration (Table 4), it is possible differences in transsulfuration were too small to detect statistically, yet large enough to modestly affect plasma cysteine concentrations. It is also possible that factors other than production rate of cysteine were affecting plasma cysteine concentrations.

Plasma concentrations of serine were decreased ($P = 0.001$) in response to Met supplementation. Decreased serine concentration in response to Met treatment could reflect the use of serine to produce cystathionine during Met transsulfuration (Awawdeh et al., 2004). Finkelstein and Martin (1986) observed an increase in the hepatic cystathionine synthase activity and a decrease in the concentrations of serine in liver of rats receiving Met. The authors suggested that increased activity of cystathionine synthase led to increased consumption of serine and homocysteine to synthesize cystathionine, which consequently results in decreased serine concentration. Also, a Met × GAA-quadratic interaction ($P = 0.01$) was observed for serine, because 7.5 g/d GAA decreased plasma serine of steers receiving no Met, but increased plasma serine for those steers supplemented with Met; we have no explanation for this effect.

Methionine supplementation decreased ($P < 0.05$) plasma concentrations of BCAA and lysine. The decreases in plasma BCAA in response to Met supplementation may have been due to increased uptake and utilization of BCAA for protein accretion when supplemental Met was provided (Campbell et al., 1997).

Plasma concentrations of ornithine were decreased ($P < 0.03$) when Met was supplemented, whereas plasma ornithine was not affected by supplemental GAA. In previous work (Ardalan et al., 2020), increases were observed for plasma arginine and ornithine for heifers receiving GAA supplementation, suggesting the arginine-sparing effect of GAA supplementation increased arginine availability, which could lead to more ornithine being formed from arginine. We did not measure plasma arginine in the current experiment, but, if arginine was spared by GAA supplementation, the effect did not carry through to affect plasma ornithine concentrations.

Conclusions

Increases in plasma creatine in association with no increase in urinary excretion of GAA in response to postruminal supplementation of GAA suggests that GAA can be an effective way to increase creatine availability to cattle. This experiment demonstrated that GAA supplementation may improve protein deposition when methyl group sources such as Met are supplemented concurrently or provided in adequate amounts by the basal diet. Because supplemental GAA elevated plasma homocysteine, the experimental model may be useful for inducing a methyl group deficiency in cattle. Supplementation of GAA did not significantly increase methylation reactions, suggesting that methylation reactions other than GAA synthesis may have been reduced by GAA supplementation. Further research is necessary to elucidate and fully understand the nature and extent of these alterations following GAA provision to cattle.

Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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Table 3-1. Composition of experimental diet

Ingredient	% of DM
Soybean hulls	82.20
Wheat straw	8.50
Cane molasses	4.18
Premix	5.12
Calcium phosphate	1.96
Sodium bicarbonate	1.25
Calcium carbonate	1.03
Magnesium oxide	0.41
Trace mineral salt[1]	0.22
Vitamin premix[2]	0.13
Sulfur	0.10
Selenium premix[3]	0.010
Bovatec-91[4]	0.018
Nutrient composition	
Crude protein	9.3
Organic matter	91.9

[1] Composition: > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.

[2] Provided (per kg diet DM): 4,950 IU vitamin A, 3,450 IU vitamin D, and 45 IU vitamin E.

[3] Provided 0.06 mg Se/kg diet DM.

[4] Supplied 36 mg lasalocid/kg diet DM (Zoetis, Florham Park, NJ).

Table 3-2. Effect of Met and GAA supplementation on DM intake (DMI), digestibilities, and nitrogen retention

	0 Met			6 g/d Met								
	GAA, g/d							*P*-value [1]				
Item	0	7.5	15	0	7.5	15	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
n	7	7	7	7	6	6						
Dietary DMI, kg/d	2.69	2.68	2.66	2.65	2.65	2.66	0.03	0.38	0.87	0.96	0.39	0.71
Digestibility[2], %												
DM	74.5	74.0	74.2	79.4	77.2	79.6	1.94	<0.01	0.97	0.15	0.76	0.31
Organic matter	77.0	75.9	76.2	80.7	79.0	81.3	1.91	<0.01	0.92	0.15	0.52	0.46
Nitrogen, g/d												
Total intake	80.7	83.3	85.7	80.3	83.7	86.8	0.60	0.47	<0.01	0.76	0.19	0.95
Diet	44.4	44.3	44.0	43.8	43.7	44.2	-	-	-	-	-	-
Infused	36.4	39.1	41.7	36.5	40.0	42.6	-	-	-	-	-	-
Urinary	32.3	35.3	37.6	29.3	30.9	31.8	1.62	0.01	<0.01	0.73	0.23	0.98
Fecal	22.0	23.2	22.8	18.2	19.9	18.0	1.59	<0.01	0.64	0.02	0.38	0.35
Retained	26.4	24.9	25.3	32.7	32.8	36.9	2.10	<0.01	0.34	0.29	0.10	0.71

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA.
[2] Excludes ruminal and abomasal infusions from calculation of digestibility.

Table 3-3. Effect of Met and GAA supplementation on urinary output and plasma concentrations of GAA, creatine, and creatinine

	0 Met			6 g/d Met								
	GAA, g/d							*P*-value [1]				
Item	0	7.5	15	0	7.5	15	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
n	7	7	7	7	6	6						
Urinary, g/d												
GAA	0.52	0.74	0.70	0.67	0.62	0.66	0.17	0.97	0.15	0.40	0.14	0.11
Creatine	5.7	7.8	7.1	6.7	6.1	6.5	1.5	0.41	0.33	0.39	0.18	0.07
Creatinine	5.7	7.9	6.9	6.3	6.6	6.9	1.6	0.61	0.13	0.14	0.55	0.13
Plasma, mg/L												
GAA	0.45	0.58	0.63	0.45	0.62	0.61	0.046	0.84	<0.01	0.02	0.73	0.43
Creatine	23.8	24.9	26.2	21.1	22.5	24.9	1.1	<0.01	<0.01	0.58	0.20	0.73
Creatinine	6.62	6.94	6.63	6.55	6.52	6.53	0.3	0.32	0.97	0.35	0.94	0.31
Renal reabsorption, %												
GAA	-50.1	-28.0	-20.5	-43.9	-12.6	-11.7	19.6	0.64	<0.01	0.22	0.90	0.67
Creatine	71.5	71.0	73.5	66.8	70.3	75.6	2.9	0.61	0.01	0.52	0.11	0.87

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA.

Table 3-4. Effect of Met and GAA supplementation on methionine flux

	0 Met			6 g/d Met								
	GAA, g/d							*P*-value[1]				
Item	0	7.5	15	0	7.5	15	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
n	6	6	6	6	5	5						
Flux (mmol Met/h)												
1-^{13}C-L-methionine	8.02	10.08	9.94	13.71	11.95	11.10	1.18	<0.01	0.73	0.71	0.04	0.38
Methyl-$^{2}H_3$-L-methionine	10.68	12.74	13.18	13.67	12.90	12.59	0.86	0.17	0.34	0.65	0.03	0.42
Metabolizable methionine[2]	1.31	1.31	1.30	2.97	2.97	2.99	0.01	<0.0001	0.75	0.69	0.21	0.53
Met deposition[3]	0.95	0.86	0.92	1.14	1.13	1.31	0.09	<0.01	0.34	0.21	0.19	0.88
Protein synthesis[4]	7.63	9.65	9.55	11.89	10.12	9.42	1.19	0.10	0.78	0.76	0.04	0.36
Protein degradation[5]	6.70	8.77	8.64	10.74	8.98	8.12	1.18	0.18	0.73	0.71	0.04	0.38
Homocysteine production[6]	2.95	3.12	3.59	1.85	2.87	2.63	0.99	0.49	0.28	0.90	0.69	0.75
Transsulfuration[7]	0.36	0.45	0.38	1.84	1.85	1.68	0.09	<0.01	0.35	0.19	0.23	0.88
Remethylation[8]	2.60	2.64	3.16	0.11	0.96	1.44	0.97	0.02	0.31	0.97	0.67	0.79

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA
[2] Metabolizable Met = ((DM intake, kg/h × 6.71 mmol metabolizable Met/kg DM intake [Campbell et al., 1997]) + Supplemental Met
[3] Met deposition = N retention, g/h × 6.25 g protein/g N × 0.134 mmol Met/g protein (Ainslie et al., 1993)
[4] Protein synthesis = PD + Met deposition
[5] Protein degradation = 1-^{13}C-L-methionine flux − Metabolizable methionine
[6] Homocysteine production (use of Met in methylation reactions) = Methyl-$^{2}H_3$-L-methionine flux – PS
[7] Transsulfuration = 1-^{13}C-L-methionine flux – PS
[8] Remethylation = Methyl-$^{2}H_3$-L-methionine flux – 1-^{13}C-L-methionine flux

Table 3-5. Effect of Met and GAA supplementation on plasma AA concentrations from blood collected on d 10

	0 Met			6 g/d Met							
	GAA, g/d							*P*-value[1]			
Amino acid, µ*M*	0	7.5	15	0	7.5	15	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
n	7	7	7	7	6	6						
Amino acid, µM												
Homocysteine	11.6	13.7	12.7	9.9	9.9	10.0	1.22	<0.01	0.26	0.10	0.36	0.10
Methionine	12.7	12.5	14.4	23.8	22.6	25.2	1.72	<0.01	0.21	0.19	0.93	0.67
Cysteine	92.8	98.3	95.8	109.7	113.5	110.4	4.82	<0.01	0.40	0.06	0.59	0.89
Ornithine	77.6	62.4	73.0	54.3	59.8	59.9	6.37	0.03	0.93	0.28	0.35	0.11
Tryptophan	34.0	30.7	35.3	28.3	30.0	30.3	2.38	0.06	0.48	0.43	0.87	0.25
Tyrosine	45.1	47.7	51.2	37.7	45.1	43.4	4.91	0.09	0.16	0.57	0.96	0.49
Threonine	120.8	129.7	112.8	96.0	124.2	128.3	16.20	0.54	0.14	0.09	0.02	0.95
Leucine	155.6	126.0	144.6	106.9	106.8	110.2	10.3	<0.01	0.60	0.05	0.33	0.09
Isoleucine	119.7	107.4	115.5	94.9	96.6	98.5	5.70	<0.01	0.95	0.22	0.41	0.22
Valine	332.4	290.6	308.8	241.1	257.2	254.8	19.3	<0.01	0.67	0.32	0.12	0.07
Lysine	120.0	102.9	123.5	89.3	95.2	106.8	8.46	0.02	0.23	0.16	0.42	0.29
Phenylalanine	61.4	56.04	60.09	58.60	58.29	62.14	5.73	0.90	0.82	0.44	0.63	0.76
Glutamate	139.9	155.0	143.4	156.4	149.4	160.6	17.33	0.58	0.71	0.81	0.97	0.22
Glutamine	201.7	196.0	232.4	191.0	224.6	202.8	21.61	0.75	0.17	0.80	0.53	0.08
Aspartic acid	4.9	5.7	5.8	5.7	6.3	6.2	0.53	0.33	0.10	0.35	0.54	0.94
Asparagine	29.1	31.5	32.2	28.5	33.0	32.0	2.42	0.90	0.12	0.32	0.91	0.60

Proline	57.7	56.8	59.7	53.5	57.7	55.8	2.55	0.27	0.42	0.80	0.94	0.29
Alanine	193.7	218.8	207.1	199.1	225.1	219.0	11.64	0.41	0.16	0.10	0.78	0.90
Serine	174.0	149.2	169.6	68.9	138.3	89.7	17.09	<0.01	0.60	0.19	0.42	0.01
Glycine	229.5	243.4	223.5	178.8	231.0	193.7	23.92	0.06	0.81	0.07	0.58	0.40

[1] G-L, Linear effect of GAA; G-Q, Quadratic effect of GAA.

Chapter 4 - Relative availability of metabolizable methionine from 2 ruminally protected sources of methionine fed to lactating dairy cattle[1]

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ABSTRACT

Our objective was to evaluate the lactational responses of dairy cows to methionine provided from 2 ruminally protected sources of methionine activity. Twenty-one Holstein dairy cows [11 primiparous (634 kg of body weight, 140 d in milk) and 10 second-parity (670 kg of body weight, 142 d in milk)] were assigned to a treatment sequence in 4 replicated 5 × 5 Latin squares plus 1 cow, with 14-d periods. Treatments were as follows: control; 7.5 or 15 g/d of a ruminally protected product of 2-hydoxy-4-methylthio-butyric acid (NTP-1401; Novus International Inc., St. Charles, MO); or 7.5 or 15 g/d of a ruminally protected dl-methionine product (Smartamine M; Adisseo, Alpharetta, GA). The diet was predicted to meet metabolizable protein and energy requirements. Diets contained 16.1% crude protein, and the control diet was predicted to be deficient in metabolizable methionine (1.85% of metabolizable protein) but sufficient in lysine (6.8% of metabolizable protein). Feed intake and milk yield were measured on d 11 to 14. Blood was collected on d 14. Dry matter intake, milk yield, energy-corrected milk, milk fat yield and percentage, and efficiencies of milk and energy-corrected milk yield were not affected by treatment. Milk protein percentage and milk protein yield increased linearly with supplementation, without differences between methionine sources or interactions between source and level. Linear regressions of milk protein percentage and milk protein yield against supplement amount within source led to slope ratios (NTP -1401: Smartamine M) of 95% for protein percentage and 84% for protein yield, with no differences between sources for increasing milk protein. Plasma methionine concentrations were increased linearly by methionine supplementation; the increase was greater for Smartamine M than for NTP-1401. Plasma d-methionine was increased only by Smartamine M. Plasma 2-hydoxy- 4-methylthio-butyric acid was increased only by NTP-1401. Our data demonstrated that supplementation with

these methionine sources can improve milk protein percentage and yield, and the 2 methionine sources did not differ in their effect on lactation performance or milk composition.

Key words: rumen protection, methionine, milk protein

INTRODUCTION

Methionine is often one of the most limiting AA for protein synthesis and optimized dairy cow production (Schwab et al., 2004; Varga, 2010). Accurate provision of Met to meet the cow's requirements can increase lactation performance (Ordway et al., 2009; Brake et al., 2013), improve the efficiency of N use, and minimize N excretion (Huhtanen and Hristov, 2009; Lee et al., 2012). Due to the high rates of degradation of AA within the rumen (Volden et al., 1998), feeding ruminally unprotected dl-Met is not considered a useful practice for ruminants (Noftsger et al., 2005). Under some circumstances, it is more efficient to provide ruminally undegraded Met as a ruminally protected methionine (**RPM**) product rather than as a ruminally undegraded protein source that provides a broad spectrum of AA rather than only Met (Broderick et al., 2009).

Dietary ingredients that could be used to increase Met supply to cows include RPM, the methionine hydroxy analog 2-hydroxy-4-(methylthio)butyric acid (**HMTBa**), and the isopropyl ester of HMTBa. Ardalan et al. (2010) reported that supplementation of RPM could improve the reproductive performance and health status of dairy cows. In a meta-analysis (Zanton et al., 2014) of supplemental dietary Met sources (such as RPM and HMTBa) or postruminal infusion of Met to dairy cows, dietary Met sources increased milk protein yield across 64 studies. Another meta-analysis (Patton, 2010) based on 35 studies in which 2 types of RPM were fed to dairy cows also indicated that adding Met to the diet increased milk protein content and yield, and increased milk yield slightly. As a consequence of the beneficial effects of Met supplementation,

such as increased milk protein content and yield, technologies have been developed and are currently being developed to prevent Met degradation by ruminal bacteria.

The main objective of this experiment was to evaluate the relative effectiveness of a ruminally protected HMTBa supplement hypothesized to provide metabolizable Met to dairy cows compared to an established ruminally protected product containing DL-Met. We hypothesized that supplementation of metabolizable Met would improve the lactational performance of dairy cows, predominantly by increasing milk protein percentage and yield. Data from Brake et al. (2013) and Osorio et al. (2013) demonstrated that change in milk protein percentage in response to RPM supplementation was a sensitive indicator for determining the effectiveness of RPM products, so we selected milk protein as our key response criteria.

MATERIALS AND METHODS

All procedures involving animals were approved by the Kansas State University Institutional Animal Care and Use Committee.

Animals and Experimental Design

Twenty-one Holstein dairy cows [11 primiparous (140 ± 16 DIM, 634 ± 45 kg of BW, 3.6 ± 0.35 BCS, mean ± standard deviation) and 10 multiparous (142 ± 15 DIM, 670 ± 46 kg of BW, 3.2 ± 0.38 BCS, all parity 2)] were housed at the Kansas State University Dairy Teaching and Research Center in tie stalls with rubber mats and wood shavings. Cows were used in 4 replicated 5 × 5 Latin squares; 2 squares contained only primiparous cows, and 2 squares contained only multiparous cows. Treatment sequences were balanced for carryover effects across the 2 squares of primiparous cows, and across the 2 squares of multiparous cows. The extra primiparous cow, which was initially included as a potential replacement but then was carried through the experiment, was given treatments in a random sequence. Treatments were

control (no supplemental Met); NTP-1401 (Novus International Inc., St. Charles, MO) supplemented at 7.5 g/d; NTP-1401 supplemented at 15 g/d; Smartamine M (Adisseo, Alpharetta, GA) supplemented at 7.5 g/d; and Smartamine M supplemented at 15 g/d.

The NTP-1401 is a ruminally protected product containing HMTBa, which cattle can convert to Met (Lapierre et al., 2011). It contains 76% DL-HMTBa, and ruminal protection is provided by a coating of copolymers and cellulose, which are sensitive to the acidic conditions of the abomasum. Smartamine M is Met coated with poly (2-vinylpyridine-co-styrene), which is sensitive to the acidic abomasal pH. Smartamine M contains 75% DL-Met, and 80% of the Met is available for intestinal absorption (Rulquin and Kowalczyk, 2003), providing 0.6 g of metabolizable Met per 1 g of Smartamine M. We hypothesized that the metabolizable Met supply from NTP-1401 was similar to that of Smartamine M, which is why we tested the same amounts of the 2 products.

Treatments were top-dressed on the TMR and hand mixed with the top third of the diet; half of each daily amount was provided at each of the 2 feedings. The diet was mixed as TMR (Table 1) once daily and added to bunks twice daily. Cows were individually fed at 0600 and 1600 h for ad libitum intake, with free access to water. Total daily feed was adjusted to allow for approximately 10% refusals. The diet is described in Table 1 and was evaluated by the Cornell Net Carbohydrate and Protein System model, version 4.0 (Fox et al., 1992; Russell et al., 1992; Sniffen et al., 1992) to meet the MP and energy requirements when DMI was 25.58 kg/d for a lactating Holstein cow producing 45 kg/d of milk with 3.50% milk fat and 3.00% milk true protein. Diets were formulated to have a moderate level of crude protein (~16%) and a predicted relative deficiency of metabolizable Met (~1.85% of MP) with sufficient levels of Lys (~6.8% of MP), resulting in Lys:Met of 3.7:1.

Each of the 5 experimental periods were 14 d long and included 10 d for adaptation to treatments and 4 d for sample and data collection. The total trial length was 70 d. Body condition score was measured on a scale of 1 to 5 (Wildman et al., 1982) by 3 trained personnel at the beginning of the trial and after each period, and BW was measured on the same days. Cows were milked 3 times per day in a milking parlor at 0700, 1500, and 2300 h, and milk yields were recorded at each milking. Animals were observed daily, and the health status of each animal was evaluated and recorded. In the last period, 1 cow from the control treatment was removed from the trial with mastitis, so 104 of 105 possible observations were collected.

Data and Sample Collection and Analysis

Daily intake was calculated from feed offered and feed refused for each cow on d 9 through 13 of each period. Samples of the TMR and feed ingredients (cottonseed, alfalfa, grain mix, and corn silage) were collected on d 9 through 13. Subsamples of the TMR and each feedstuff were dried in a forced-air oven at 55°C for 24 h to determine partial DM. Dried samples of the TMR and of each feedstuff were mixed within period to obtain composite samples and stored for subsequent analysis. Samples of the TMR from each period, as well as samples of each feedstuff from each period, were sent to Cumberland Valley Analytical Services (Hagerstown, MD) for all analyses except AA analysis. Analyses were conducted for the following (methods in parentheses): DM (AOAC International, 2000; method 930.15); CP (AOAC International, 2000; method 990.03); soluble protein (Krishnamoorthy et al., 1982); ADF (AOAC International, 2000; method 973.18), with acid detergent insoluble CP measured as CP remaining in the residue; NDF (Van Soest et al., 1991; with use of heat-stable amylase), with neutral detergent insoluble CP measured as CP remaining in the residue; in vitro NDF digestibility (Goering and Van Soest, 1970); ADL (Goering and Van Soest, 1970); ethanol-

soluble carbohydrates (Hall, 2000); starch (Hall, 2009; corrected for free glucose); crude fat (AOAC International, 2006; method 2003.05); ash (AOAC International, 2000; method 942.05); sulfur by combustion (Leco S632 Sulfur Combustion Analyzer; Leco Corporation, St. Joseph, MI); chloride by extraction with 0.5% nitric acid, followed by analysis with potentiometric titration with silver nitrate (Metrohm 848 Titrino Plus Metrohm USA Inc., Riverview, FL); remaining minerals (AOAC International, 2000; method 985.01) using a Perkin Elmer 5300 DV ICP (Perkin Elmer, Shelton, CT). Amino acids were analyzed by HPLC as described below, following acid hydrolysis (6 *M* HCl, 22 h, 105°C). Methionine and cysteine were analyzed as methionine sulfone and cysteic acid following performic acid oxidation and acid hydrolysis (Moore, 1963). In the final 4 d of each period, samples of TMR were analyzed for particle size with a Penn State Particle Separator (Kononoff et al., 2003).

Milk samples (25 mL) from individual cows were collected during the final 4 d of each period at each milking, preserved with 2-bromo-2-nitropropane-1,3 diol, and stored at 4°C for analysis of milk components. Milk samples from each cow for each milking were analyzed for fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments Inc., Chaska, MN), MUN (MUN spectrophotometer; Bentley Instruments Inc.), and somatic cells (SCC 500; Bentley Instruments Inc.) at the Heart of America DHIA (Manhattan, KS). The yield of ECM was calculated as ECM = [(0.327 × milk, kg/d) + (12.95 × fat, kg/d) + (7.65 × protein, kg/d)], and FCM (3.5%) was calculated as FCM = [(0.4324 × milk, kg/d) + (16.216 × milk fat, kg/d)] (Tyrrell and Reid, 1965; Dairy Records Management Systems, 2014).

Blood samples were collected from the coccygeal vein of each cow by venipuncture into evacuated tubes (9 mL) containing K_3-EDTA (Vacuette; Greiner Bio-One GmbH, Kremsmunster, Austria) at 4 h after the afternoon feeding on the final day of each period.

Immediately after collection, samples were stored on crushed ice and then centrifuged at 4°C for 15 min at 3,000 × *g* to harvest plasma. Plasma samples were frozen at −20°C for later analyses of BHB (Kit #H7587–58; Pointe Scientific Inc., Canton, MI), nonesterified fatty acids (NEFA-HR; Wako Chemicals USA Inc., Richmond, VA), urea-N, glucose, and AA concentrations. Plasma urea (Marsh et al., 1965) and plasma glucose (Gochman and Schmitz, 1972) were measured with an AutoAnalyzer (Technicon Analyzer II; Technicon Industrial Systems, Buffalo Grove, IL). Plasma free AA were determined by HPLC after deproteinization by mixing plasma with equal volumes of 10% (wt/vol) sulfosalicylic acid containing norleucine as an internal standard (Campbell et al., 1997). Chromatography was achieved on a Li cation-exchange column (4.0 × 100 mm; Pickering Laboratories Inc., Mountain View, CA). After elution from the column, AA were derivatized with *o*-phthalaldehyde before fluorescence was measured, and AA were quantified with reference to an internal standard (100 m*M* norleucine; Brake et al., 2013). Plasma concentrations of L- and D-Met and of HMTBa were analyzed following deproteinization with acetonitrile. Both L- and D-Met were analyzed by LC-MS/MS using a chiral column on a Prominence HPLC (Shimadzu, Columbia, MD) with an API 4000 MS detector (AB Sciex; Concord, ON, Canada). Isotope dilutions of $^{13}C_5$-^{15}N-L-Met and $^{2}H_3$-D-Met were used for quantification. The HMTBa was analyzed by LC-MS/MS using the same HPLC equipment with a hydrophilic liquid interaction chromatography column; tolbutamide was used as an internal standard.

Data were analyzed as a Latin square using PROC MIXED in SAS (version 9.3; SAS Institute, Cary, NC) with parity, period, treatment, and parity × treatment as fixed effects, and with cow as a random effect. Treatment means were separated with pre-planned, nonorthogonal contrasts including: the effect of Met source (source); the Met source × level interaction (source

× level); the linear effect of Met level (level linear); and the quadratic effect of Met level (level quadratic). The contrast coefficients are provided in Table 2. These contrasts were selected because they allowed assessment of the nature of the response to supplemental methionine (level linear and level quadratic contrasts); the differences between Met sources (source contrast); and whether differences between Met sources were dependent on the level of product provided (source × level contrast).

Linear regressions were conducted for milk protein percentage and milk protein yield, with these variables regressed against supplement amount within source. Regressions were conducted using PROC MIXED in SAS with a model including parity, period, and supplementation level within source as fixed effects, and cow as a random effect.

RESULTS AND DISCUSSION

Experimental Diet and Design

Diets were formulated to have a predicted deficiency of metabolizable Met but sufficient levels of Lys. The nutrient composition of the TMR and of dietary ingredients are presented in Tables 1 and 3. The concentration of CP averaged 16.1% of dietary DM, and the dietary AA profile was similar to expectations, given the dietary ingredients. The particle size of the TMR was measured, and 10.6 ± 1.7%, 33.0 ± 3.4%, 40.1 ± 3.6%, and 16.4 ± 3.0% of the TMR remained on the top, second, third, and bottom sieves, respectively, of a Penn State Particle Separator (Kononoff et al., 2003).top, second, third, and bottom sieves, respectively, of a Penn State Particle Separator (Kononoff et al., 2003).

All cows were fed the same basal diet, so any effects of diet—such as particle size, forage level, and total protein concentration—on treatment groups were the same. Our basal diet was designed to be most limiting in Met so that responses to supplemental Met could be readily

measured. The ability of lactating cows to respond to supplementation with RPM depends on Met status, the availability of other essential AA, and the availability of Met from RPM sources. Although parity and stage of lactation could alter responses to RPM, these factors were balanced across all treatments. Moreover, we observed no evidence of treatment × parity interactions ($P \geq 0.10$) for any criterion except percentage SNF ($P = 0.03$); thus, the treatments are discussed without consideration of parity (primiparous vs. multiparous). Davidson et al. (2008) studied the responses of primiparous and multiparous cows to RPM, and they observed that supplementation of RPM increased milk protein yield in multiparous cows but not in primiparous cows. The nonresponsiveness of primiparous cows to RPM likely reflected the low milk protein yield of primiparous cows, which was only 73% of that for multiparous cows (Davidson et al., 2008). In our study, primiparous cows had milk protein yields that were 95% of multiparous cows, which likely explains the similar responses to RPM between our primiparous and multiparous cows.

DMI and Lactation Responses

Findings for DMI, milk yield, and milk composition are shown in Table 4. We found that DMI was not affected by Met supplementation ($P \geq 0.14$). Using meta-analyses, Zanton et al. (2014) observed different effects of Met sources on DMI. Dry matter intake was increased for cows supplemented with Smartamine, but reductions in DMI were noted for cows supplemented with Mepron. Moreover, they observed no treatment differences in DMI when cows were fed HMTBa or infused with dl-Met. Zanton et al. (2014) suggested that consumption of excessive levels of Met can have a negative effect on DMI. Because our levels of Met supplementation were modest, our observation that intake was unaffected by treatment was not surprising.

In the current study, milk yield averaged 46.2 kg/d and was not affected by the 2 Met sources. Two meta analyses have suggested that supplementing metabolizable Met to lactating

dairy cattle does not typically lead to large changes in milk yield. Zanton et al. (2014) found no overall effect of supplemental Met on milk yield, but did find small differences among Met sources. Patton (2010) observed a slight increase in milk yield when RPM were supplemented, but the response differed among cattle breeds and among predominant dietary forages.

In general, the major effect of adding RPM to the diet of dairy cattle is often an increase in milk protein concentration, yield, or both (Patton, 2010; Zanton et al., 2014), so we selected milk protein percentage and milk protein yield as key response criteria for evaluating responses to the 2 products in our trial. In our experiment, milk protein percentage and milk protein yield increased linearly with Met supplementation ($P < 0.01$), and we found no differences between sources. We found a tendency ($P = 0.10$) for an interaction between source and level for milk protein percentage, because milk protein percentage was slightly greater for NTP-1401 than for Smartamine M when 7.5 g/d of product was fed, but it was slightly greater for Smartamine M than for NTP-1401 when 15 g/d of product was fed. The increases in milk protein percentage and yield indicate that Met increased mammary protein synthesis and that the tested supplements were generally similar in their ability to provide metabolizable Met to cows. A meta-analysis (Patton, 2010) reported that the average responses of lactating cows to RPM were increases of 0.07% in milk protein percentage and 27 g/d in milk protein yield. As well, Zanton et al. (2014)—using a mixed model, weighted meta-analysis—showed that milk protein yield responses ranged from 13 to 35 g/d when different Met sources were supplemented.

We used the linear regressions of milk protein percentage and milk protein yield against supplement amount within source to directly compare the 2 RPM sources. Slope ratios (NTP - 1401: Smartamine M) were 0.95:1 for protein percentage ($P = 0.65$ for the difference from 100%) and 0.84:1 for protein yield ($P = 0.60$ for the difference from 100%), suggesting no

differences between sources for increasing milk protein (Figure 1a and b). Zanton et al. (2014) demonstrated an increase across all Met sources of 2.23 g of milk protein yield per gram of metabolizable Met. Our slopes demonstrated that milk yield responses in our study were 3.1 and 2.6 g of milk protein per gram of RPM product for Smartamine M and NTP-1401, respectively. Assuming that the metabolizable Met supplies were 60% of product weights, our responses were about twice the average reported by Zanton et al. (2014); our responses in milk protein yield were probably greater than the average from the analysis of Zanton et al. (2014) because our research model was specifically designed to create a Met-limiting condition.

We observed no effect of RPM on milk fat yield or milk fat percentage (1.60 kg/d and 3.49%), in agreement with observations from the literature. Using a meta-analysis, Patton (2010) observed that RPM provided as Mepron increased milk fat yield, whereas RPM provided as Smartamine did not affect milk fat yield. In contrast, the meta-analysis of Zanton et al. (2014) concluded that RPM provided as either Mepron or Smartamine increased milk fat yield, and that even greater increases in milk fat yield were observed when HMTBa was provided without protection from ruminal degradation. These results suggest that provision of both ruminally available HMTBa and post-ruminally available Met may affect milk fat yield, although we did not observe any differences in milk fat concentration or yield in our study.

Increases in milk fat in response to Met supplementation can occur due to synthesis of choline from Met, which is a way to increase transport of lipids as very low density lipoproteins (McCarthy et al., 1968; Auboiron et al., 1995), due to ruminal effects via improved protozoal growth in the rumen, which can alter production of bioactive fatty acids in milk (Patton et al., 1970; Huws et al., 2012). Highly fermentable diets containing large amounts of unsaturated fatty acids diet can inhibit milk fat synthesis via alteration in ruminal biohydrogenation pathways and

increases in ruminal outflow of bioactive fatty acids (Baldin et al., 2018). Baldin et al. (2018) studied the effects of HMTBa on milk fat synthesis in cows with experimental milk fat depression (low dietary NDF plus addition of soybean oil to the diet). Milk fat percentage was increased by HMTBa supplementation in the cows with induced milk fat depression, and the authors attributed the improved milk fat response with HMTBa to improved rumen microbial composition or growth, which stabilized ruminal biohydrogenation, thus preventing changes in biohydrogenation pathways and the related milk fat depression. Because milk fat concentrations demonstrated that our cows were not experiencing milk fat depression, a change in milk fat would not be expected as a response to ruminally available Met. Moreover, the milk protein responses elicited by both RPM sources suggest that both sources were resistant to ruminal degradation and would be unlikely to affect the rumen microbial community.

We observed no treatment differences for ECM, FCM, or efficiencies of milk, ECM, and FCM production. Lactose yield was unaffected by treatment, but milk lactose percentage was decreased slightly by Met supplementation ($P < 0.001$). The effect was somewhat greater for Smartamine M than for NTP-1401 at the low level of supplementation, but somewhat greater for the NTP-1401 than for Smartamine M at the high level of supplementation (source × level interaction, $P = 0.02$). The SNF percentage was unaffected by NTP-1401, but demonstrated a quadratic effect for Smartamine M, being decreased by the low level of Smartamine M but increased by the high level of Smartamine M (quadratic effect of Met, $P = 0.01$; source × level interaction, $P = 0.04$). Yield of SNF was not affected by treatment ($P \geq 0.21$). Neither MUN nor SCC was affected by treatment ($P \geq 0.14$).

BW and BCS

Initial BW averaged 662 kg and were not different among treatments ($P \geq 0.24$; Table 4). Initial BCS averaged 3.2 and were not different among treatments ($P \geq 0.28$). Because we used a Latin square experimental design, no differences in initial BW or BCS were expected.

Plasma AA and Metabolites

The effect of NTP-1401 and Smartamine M on plasma concentrations of L-Met, D-Met, and HMTBa are shown in Table 5. Plasma l-Met was increased linearly ($P = 0.04$) by both RPM sources without differences between the Met sources ($P = 0.61$). However, we found a tendency ($P = 0.08$) for greater increases in plasma l-Met with NTP-1401 than with Smartamine M at the low level (7.5 g/d) of supplementation, but greater increases with Smartamine M than with NTP-1401 at the high level (15 g/d) of supplementation.

Supplementation with Smartamine M increased plasma D-Met, but no plasma samples from cows fed NTP-1401 contained measurable concentrations of D-Met (Table 5; source × level interaction, $P < 0.001$). Cows receiving NTP-1401 had elevated plasma concentrations of HMTBa, whereas none of the cows fed Smartamine M had measurable concentrations of HMTBa (Table 5; source × level interaction, $P < 0.001$). These results were expected, because Smartamine M and NTP-1401 contain DL-Met and HMTBa, respectively. Relatively few measurements of plasma D-Met concentrations in dairy cattle have been reported in the literature. Lapierre et al. (2012) abomasally infused 15 g/d of DL-Met and observed arterial concentrations of 13.8 μ*M* D-Met, which proportionately exceeded our observed concentration of 3.5 μ*M* when 9 g/d metabolizable DL-Met was provided by 15 g/d Smartamine M. Similarly, plasma concentrations of HMTBa are scarce in the literature, but our concentration of 4.5 μ*M* when 15 g/d NTP- 1401 was fed was somewhat lower, proportional to supply, than the plasma

HMTBa concentration of 35 μ*M* found in lactating cows receiving abomasal infusions of 36 g/d of HMTBa (Lapierre et al., 2011). We speculate that our somewhat lower plasma concentrations of both D-Met and HMTBa might have been due to the greater production levels of our cows compared with those of Lapierre et al. (2011, 2012).

Although only the l-isomer of Met is used for protein synthesis, cattle are capable of converting D-Met (Lapierre et al., 2012) and HMTBa (Lapierre et al., 2011) to L-Met, and Campbell et al. (1996) showed that D-Met supplementation improved protein deposition in Met-deficient growing steers. However, D-Met is not converted rapidly to L-Met; L-Met removal rates were estimated to be 6-fold faster than those for D-Met, demonstrating a slower metabolism rate for D-Met than L-Met (Lapierre et al., 2012). This finding explains the greater plasma concentrations observed when D-Met, rather than L-Met, is supplemented (Campbell et al., 1996; Lapierre et al., 2012).

Rumen-protected Met and HMTBa (Lapierre et al., 2011) can supply the Met required by dairy cows to improve lactational performance. Although HMTBa is not an AA, it is metabolized to Met. In ruminants, metabolism sites of HMTBa include the ruminal and omasal epithelia, liver, and kidney, but the synthesis rates of Met were greatest in the liver and kidney (Lobley et al., 2006). Lapierre et al. (2011) observed that HMTBa increased whole-body Met availability and, at the level of supplementation provided, 15% of Met in milk protein and 3.8 mmol/h of Met flow through plasma were provided by intravenously infused HMTBa, demonstrating that, as confirmed by our study, HMTBa can act as a source of Met for cattle.

Plasma concentrations of total Met (Table 5) were similar to values obtained as the sum of L-Met and D-Met. This result was expected and supports the accuracy of the methodologies used for their measurement.

Plasma Met concentrations were increased linearly by Met supplementation ($P < 0.001$), with differences between sources ($P < 0.001$). The increases in plasma Met were greater for Smartamine M than for the NTP-1401 (source × level interaction, $P = 0.004$), which was expected because the greatest increases in plasma Met resulted from increases in D-Met provided by Smartamine M due to the longer half-life of D-Met compared with L-Met. Increases in plasma AA concentrations are often used as an indicator of intestinal supply of AA (Rulquin and Kowalczyk, 2003; Whitehouse et al., 2017), but in the current study, because of differences in the sources of Met activity (i.e., HMTBa vs. DL-Met), it was not possible to use plasma Met to compare bioavailabilities between the products. However, the increases in plasma Met concentrations were at least indicative of significant amounts of metabolizable Met being supplied to the cows from both products.

Taurine, an end product of Met metabolism, was increased linearly by Met supplementation ($P = 0.02$). We found some differences between the 2 Met sources in the plasma taurine response (source × level, $P = 0.03$); the NTP-1401 at the low level of supplementation and Smartamine M at the high level led to greater increases in plasma taurine than the high level of NTP-1401 and the low level of Smartamine M.

Plasma concentrations of Val, Leu, Phe, Tyr, and Ser were all decreased linearly by Met supplementation ($P \leq 0.03$), perhaps reflecting increased uptake of these AA to support milk protein yield. Plasma Ser concentrations have often been observed to decrease when Met is supplemented (Campbell et al., 1996; Loest et al., 2002), and this has been attributed to the consumption of Ser during transsulfuration, which would be expected to increase with Met supply. Plasma concentrations of Leu, Thr, Ser, and Asn demonstrated source × level interactions ($P < 0.05$) because NTP-1401 at the high level and Smartamine M at the low level

led to lower concentrations than NTP-1401 at the low level and Smartamine M at the high level; we have no explanation for these interactions between source and level.

Plasma nonesterified fatty acids demonstrated a quadratic response to Met supplementation, with the low level of supplementation leading to slight decreases in concentrations (quadratic, $P = 0.05$; Table 6). Plasma BHB was not affected by treatment ($P \geq 0.16$). Although Met plays an important role in lipid metabolism and energy homeostasis in transition cows through elevated synthesis of very-low-density lipoproteins, improved hepatic gluconeogenesis, and reduced ketone body production (Ardalan et al., 2010, 2011), cows averaged 141 DIM at the initiation of our study, so it is not surprising that nonesterified fatty acids and BHB were not greatly different among treatments.

Plasma glucose demonstrated differences between Met sources; NTP-1401 slightly decreased plasma glucose concentrations, and Smartamine M slightly increased concentrations (source, $P < 0.01$), but the magnitude of change in plasma glucose was not large. Plasma urea concentrations were not affected by treatment ($P \geq 0.13$), in agreement with observations for MUN. Consistent with our observations, other authors have reported that feeding RPM did not change plasma urea concentrations (Overton et al., 1998; Blum et al., 1999; Bateman et al., 1999). Although RPM supplementation increased milk protein secretion, which might be expected to decrease PUN and MUN, the change in milk N was a very small percentage of N intake, which would make detection of changes in urea concentrations difficult.

CONCLUSIONS

Milk protein percentage and yield were increased by RPM supplementation, and the increases were generally similar for Smartamine M and NTP-1401. Slope ratio analyses suggested no significant difference between the 2 ruminally protected sources of Met in their

ability to increase milk protein yield. When Met supplementation is warranted because of limitations in the dietary supply of Met, either product can be used to enhance performance in lactating dairy cattle.

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Table 4-1. Ingredient composition of the diet

Ingredient	% of DM
Corn silage	29.9
Alfalfa	23.9
Whole cottonseed	3.9
Finely rolled corn	20.1
Solvent soybean meal	3.4
SoyBest[1]	1.8
Soybean hulls	8.1
Dried molasses	2.6
Blood meal	1.5
Energy Booster 100[2]	2.1
Limestone	0.47
Dicalcium phosphate	0.55
Salt	0.39
Trace-mineralized salt[3]	0.18
Sodium bicarbonate	0.59
Magnesium oxide	0.20
Copper sulfate	0.0053
Zinc oxide	0.0089
Selenium premix, 600 mg Se/kg	0.030
Vitamin A premix, 30,000 IU/g	0.018
Vitamin D premix, 20,000 IU/g	0.0053
Vitamin E premix, 44 IU/g	0.18
Ethylenediamine dihydriodide, 4.4%	0.0008
Rumensin 90[4]	0.0075
Yeast[5]	0.055
Biotin premix, 220 mg biotin/kg	0.11

[1] Mechanically extracted soybean meal with soy lecithins added during manufacture (SoyBest, West Point, NE).
[2] Rumen bypass fat with 98% fatty acids (Milk Specialities, Eden Prairie, MN).
[3] Composition: > 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co.
[4] Provided 15 mg monensin/kg diet DM (Elanco Animal Health, Indianapolis, IN).
[5] Diamond V XPC Yeast (Diamond V Mills, Inc., Cedar Rapids, IA).

Table 4-2. Coefficients used to generate contrasts for evaluation of treatment differences[1]

	Dietary treatment				
		NTP-1401(g/d)		Smartamine M (g/d)	
Contrast	Control	7.5	15	7.5	15
Source	0	1	1	-1	-1
Source × level	0	1	-1	-1	1
Level linear	2	0	-1	0	-1
Level quadratic	2	-2	1	-2	1

[1]NTP-1401 = ruminally protected form of 2-hydoxy-4-methylthio-butyric acid (Novus International Inc., St. Charles, MO); Smartamine M= ruminally protected DL-methionine product (Adisseo, Alpharetta, GA).

Table 4-3. Nutrient composition of the TMR and of dietary ingredients (means from samples from 5 periods with SD)

Nutrient [1]	TMR	SD	Corn silage	SD	Alfalfa	SD	Grain mix [2]	SD	Cotton seed	SD
Dry matter, %	58.7	1.3	32.0	1.3	86.4	2.0	88.3	0.5	88.9	0.3
Crude protein, % DM	16.1	0.52	8.9	0.34	19.0	1.34	18.3	0.48	22.3	0.42
Soluble protein, % DM	5.0	0.22	5.7	0.24	7.1	0.40	3.4	0.33	6.1	1.09
ADICP, % DM	0.90	0.052	0.72	0.04	1.43	0.103	0.71	0.12	1.89	0.25
NDICP, % DM	1.81	0.61	0.89	0.04	2.62	0.57	1.44	0.27	3.05	1.87
ADF, % DM	23.0	1.47	23.4	1.62	38.4	1.62	11.0	1.16	33.7	1.72
aNDF, % DM	33.3	2.06	39.3	1.87	46.0	1.52	18.0	1.41	44.0	2.50
24-h in vitro NDF digestibility, % of DM	16.5	1.31	20.9	2.35	14.7	2.94	14.1	1.21	2.6	1.50
ADL, % DM	3.8	0.54	2.8	0.49	8.9	0.85	1.3	0.18	9.1	0.69
Ethanol soluble CHO, % DM	5.0	0.33	0.9	0.17	5.5	0.58	7.2	0.48	6.0	0.53
Starch, % DM	21.3	2.07	32.7	1.84	1.2	0.57	38.8	1.48	0.7	0.35
Non-structural CHO, % DM [3]	26.3	1.98	33.6	1.89	6.7	0.95	46.1	1.52	6.7	0.88
Non-fiber CHO, % DM [4]	38.9	0.88	44.4	1.68	26.1	1.44	49.8	1.00	13.8	2.32
7-h in vitro starch digestibility, %	63.1	2.75	72.1	4.04	—	—	60.3	4.58	—	—
Crude fat, % DM	5.0	0.10	3.6	0.16	1.4	0.12	7.1	0.14	18.6	0.66
TDN, % DM [5]	71.5	0.96	72.1	1.19	56.8	1.68	83.2	0.51	86.9	0.90
NE_L, Mcal/kg [5]	1.65	0.02	1.65	0.02	1.28	0.04	1.94	0.01	2.09	1.99
DCAD, meq/100 g DM [6]	23.5	1.91	8.7	5.08	40.2	6.50	27.5	4.16	13.6	1.92
Ash, % DM	8.4	0.80	4.8	0.15	10.2	0.54	8.3	0.33	4.4	0.36
Calcium, % DM	0.85	0.06	0.23	0.07	1.16	0.048	1.15	0.12	0.17	0.010
Phosphorus, % DM	0.38	0.01	0.27	0.01	0.32	0.019	0.66	0.059	0.62	0.095
Magnesium, % DM	0.31	0.02	0.20	0.05	0.27	0.040	0.48	0.028	0.40	0.039
Potassium, % DM	1.33	0.11	1.03	0.25	2.20	0.21	1.13	0.046	1.19	0.079
Sulfur, % DM	0.19	0.01	0.12	0.01	0.21	0.024	0.21	0.014	0.25	0.012

Sodium, % DM	0.38	0.004	0.02	0.01	0.03	0.008	0.94	0.11	0.01	0.002
Chloride, % DM	0.55	0.03	0.39	0.07	0.15	0.042	1.04	0.16	0.07	0.005
Iron, mg/kg DM	475	97	70	4.96	453	134	496	29	55	6.7
Manganese, mg/kg DM	35	3.0	18	3.97	33	3.97	38	3.66	17	2.06
Zinc, mg/kg DM	83	13	20	1.47	24	2.42	161	29	39	3.54
Copper, mg/kg DM	18	1.0	6	0.49	7	0.89	37	8.62	9	0.75

AA, % of DM		
Cysteine	0.23	0.028
Aspartate	1.61	0.081
Threonine	0.59	0.068
Serine	0.74	0.048
Glutamate	1.93	0.160
Glycine	0.71	0.050
Alanine	0.91	0.046
Valine	0.72	0.028
Methionine	0.21	0.024
Isoleucine	0.29	0.015
Leucine	0.63	0.040
Tyrosine	0.74	0.035
Phenylalanine	0.73	0.026
Histidine	0.74	0.078
Lysine	0.45	0.044
Arginine	0.74	0.044
Total AA	11.96	0.67

[1] ADICP = acid detergent insoluble CP; aNDF = NDF analysis included use of heat stable amylase; CHO = carbohydrates; NDICP = neutral detergent insoluble CP.
[2] Grain mix included all ingredients except alfalfa, corn silage, and whole cottonseed.
[3] Non-structural CHO calculated as ethanol soluble CHO + starch.
[4] Non-fiber CHO calculated as 100% - CP – (aNDF – NDICP) - crude fat – ash.
[5] TDN was calculated using Equation 14 from Weiss et al. (1992). NE_L was calculated as (TDN × 0.0245) – 0.12.
[6] DCAD was calculated as Na + K – (2 × S) – Cl.

Table 4-4. Effect of supplementation with NTP-1401 or Smartamine M on milk yield and composition, BW, and BCS

	Dietary treatment[1]						*P*-value[3]			
		NTP-1401 (g/d)		Smartamine M (g/d)						
Item	Control	7.5	15	7.5	15	SEM[2]	Source	Source × level	Level linear	Level quadratic
n	20	21	21	21	21					
DMI, kg/d	27.2	27.0	26.8	27.0	27.2	0.43	0.54	0.39	0.59	0.74
Milk yield, kg/d	46.4	46.4	46.0	46.1	46.4	1.1	0.99	0.29	0.72	0.89
ECM, kg/d	45.6	45.9	45.9	45.7	46.2	0.95	0.83	0.55	0.35	0.94
3.5% FCM, kg/d	45.9	46.0	46.0	45.9	46.2	0.99	0.82	0.71	0.72	0.97
Milk:DMI	1.71	1.72	1.72	1.71	1.71	0.029	0.38	0.82	0.67	0.80
ECM:DMI	1.68	1.70	1.71	1.70	1.70	0.025	0.67	0.78	0.14	0.77
FCM:DMI	1.69	1.70	1.72	1.70	1.70	0.026	0.66	0.68	0.33	0.75
Fat, %	3.46	3.48	3.51	3.50	3.50	0.094	0.94	0.71	0.35	0.84
Fat yield, kg/d	1.59	1.60	1.61	1.61	1.61	0.041	0.77	0.99	0.53	0.99
Protein, %	2.77	2.82	2.86	2.81	2.87	0.041	0.99	0.10	<0.001	0.94
Protein yield, kg/d	1.28	1.30	1.31	1.29	1.33	0.026	0.92	0.14	0.004	0.84
Lactose, %	4.99	4.96	4.94	4.95	4.96	0.029	0.65	0.02	<0.001	0.02
Lactose, kg/d	2.31	2.30	2.27	2.28	2.30	0.052	0.89	0.18	0.26	0.59
SNF, %	8.72	8.72	8.73	8.69	8.76	0.059	0.89	0.04	0.11	0.01
SNF, kg/d	4.03	4.03	4.01	4.00	4.06	0.084	0.92	0.21	0.98	0.53
MUN, mg/dL	13.84	13.95	13.59	13.73	13.59	0.27	0.41	0.42	0.14	0.30
SCC, per uL	30	27	47	33	39	13	0.85	0.37	0.22	0.36
Initial BCS	3.21	3.22	3.22	3.18	3.19	0.10	0.28	0.84	0.82	0.84
Initial BW, kg	661	663	659	663	662	11.2	0.57	0.48	0.91	0.24

[1] NTP-1401 is a ruminally protected product containing 2-hydroxy-4-(methylthio)butyric acid. Smartamine M = ruminally protected DL-methionine product (Adisseo, Alpharetta, GA).
[2] For n = 20.
[3] Contrast coefficients are presented in Table 2.

Table 4-5. Effect of supplementation with NTP-1401 or Smartamine M on plasma concentrations of AA and 2-hydroxy-4-(methylthio)butyric acid (HMTBa)

	Dietary treatment[1]						*P*-value[3]			
		NTP-1401 (g/d)		Smartamine M (g/d)						
Amino acid, µ*M*	Control	7.5	15	7.5	15	SEM[2]	Source	Source × level	Level linear	Level quadratic
n	20	21	21	21	21					
Met (total)	19.5	20.8	20.5	22.2	25.9	0.8	<0.001	0.004	<0.001	0.81
L-Met	19.0	20.6	20.4	19.0	21.3	0.79	0.61	0.08	0.04	0.84
D-Met	0.0	0.0	0.0	1.3	3.5	0.28	<0.001	<0.001	<0.001	0.44
HMTBa	0.0	2.1	4.5	0.0	0.0	0.14	<0.001	<0.001	<0.001	0.44
Taurine	42.4	46.2	44.3	44.4	48.9	2.2	0.32	0.03	0.02	0.56
Lys	83.8	85.0	84.0	77.2	86.8	3.9	0.42	0.09	0.68	0.23
Arg	80.2	78.6	78.9	72.7	79.4	3.1	0.30	0.21	0.74	0.10
Ala	303.2	305.2	278.8	297.5	291.8	11.1	0.74	0.21	0.08	0.36
Val	399.1	391.9	377.4	372.7	382.4	11.6	0.32	0.09	0.03	0.29
Leu	129.1	125.2	119.1	116.3	122.1	4.3	0.33	0.05	0.03	0.15
Ile	84.2	83.2	79.7	76.6	80.6	2.8	0.19	0.09	0.15	0.27
Thr	123.7	124.3	115.6	116.6	120.2	4.2	0.59	0.04	0.12	0.89
Phe	68.4	64.9	60.6	60.6	63.1	2.4	0.68	0.12	0.02	0.26
Tyr	127.1	118.3	114.1	109.1	112.6	4.8	0.18	0.33	0.007	0.09
Trp	89.5	89.5	86.8	82.5	90.4	4.0	0.63	0.13	0.85	0.37
His	77.2	78.6	77.8	76.9	78.8	1.7	0.80	0.34	0.52	0.99
Ser	91.0	89.2	81.2	82.3	85.3	2.8	0.50	0.01	0.004	0.48
Gly	271.7	269.0	257.1	261.2	271.2	10.5	0.68	0.16	0.44	0.70
Asp	10.1	10.1	9.8	9.7	10.6	0.4	0.59	0.08	0.81	0.48
Asn	59.0	60.3	54.4	52.4	57.9	2.6	0.37	0.02	0.35	0.60
Glu	64.9	61.8	69.0	62.4	59.5	7.2	0.52	0.47	0.94	0.71
Gln	209.4	217.9	202.0	200.9	212.1	8.1	0.64	0.07	0.80	0.86
Cit	91.8	92.8	93.6	90.5	95.1	4.0	0.84	0.33	0.32	0.44
Orn	43.7	44.8	44.1	40.9	47.6	2.1	0.94	0.02	0.26	0.17

[1] NTP-1401 = ruminally protected form of 2-hydoxy-4-methylthio-butyric acid (Novus International Inc., St. Charles, MO); Smartamine M = ruminally protected DL-methionine product (Adisseo, Alpharetta, GA).
[2] For n = 20.
[3] Contrast coefficients are presented in Table 2.

Table 4-6. Effect of supplementation with NTP-1401 or Smartamine M on plasma metabolites

	Dietary treatment[1]						*P*-value[3]			
		NTP-1401 (g/d)		Smartamine M (g/d)						
Metabolite	Control	7.5	15	7.5	15	SEM[2]	Source	Source × level	Level linear	Level quadratic
n	20	21	21	21	21					
NEFA[4], µmol/L	94.2	85.2	97.1	88.0	89.6	4.5	0.53	0.17	0.85	0.05
BHB, µmol/L	729	782	721	717	686	36	0.16	0.68	0.56	0.32
Glucose, mmol/L	3.49	3.41	3.47	3.54	3.58	0.052	0.004	0.91	0.49	0.41
Urea, mmol/L	5.05	5.18	5.19	5.05	5.01	0.13	0.13	0.79	0.65	0.66

[1] NTP-1401 = ruminally protected form of 2-hydoxy-4-methylthio-butyric acid (Novus International Inc., St. Charles, MO); Smartamine M = ruminally protected DL-methionine product (Adisseo, Alpharetta, GA).
[2] For n = 20.
[3] Contrast coefficients are presented in Table 2.
[4] Non-esterified fatty acids.

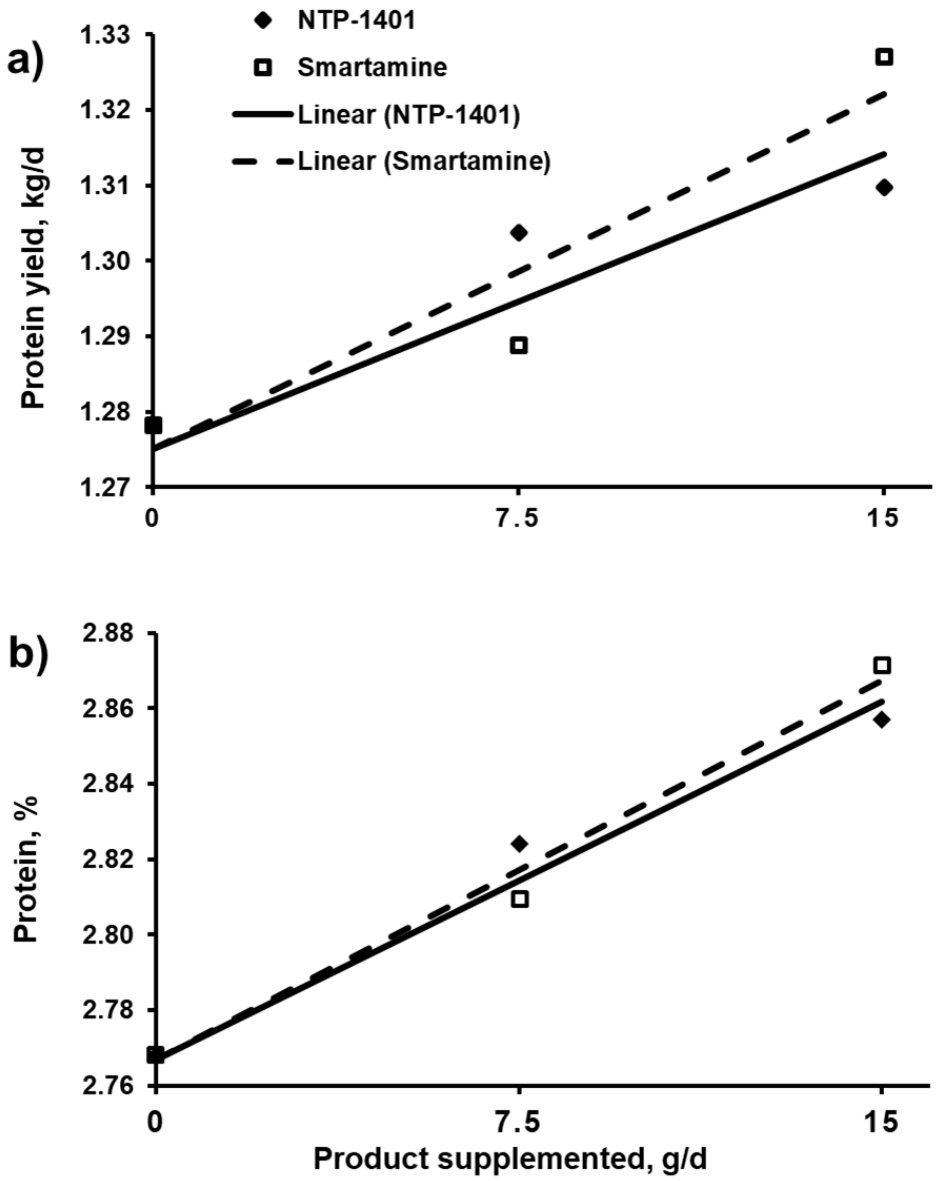

Figure 4.1. Linear regressions for (a) milk protein yield (NTP-1401 slope = 0.0026 ± 0.0010; Smartamine M slope = 0.0031 ± 0.0010; difference between slopes, $P = 0.60$) and (b) milk protein percentage (NTP-1401 slope = 0.0064 ± 0.0008; Smartamine M slope = 0.0067 ± 0.0008; difference between slopes, $P = 0.65$) in lactating dairy cows supplemented with 1 of the 2 products in amounts of 0, 7.5, or 15 g/d. NTP-1401 is a ruminally protected form of 2-hydoxy-4-methylthio-butyric acid (Novus International Inc., St. Charles, MO); Smartamine M is a ruminally protected DL-methionine product (Adisseo, Alpharetta, GA)..

Chapter 5 - Effect of post-ruminal casein infusion on milk yield and composition and efficiency of nitrogen use in dairy cows

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Abstract

Adequate supply of protein and amino acids can improve nitrogen utilization efficiency. Casein is the predominant protein found in milk; therefore, casein supplementation can improve protein synthesis and nitrogen efficiency in the body by increasing amino acid supply. An experiment was conducted to evaluate the effect of post-ruminal supplementation of casein on milk yield and composition and whole body protein deposition. Two ruminally cannulated Holstein dairy cows (599 ± 17 kg initial BW) were used in switch-back design. Cows were fed twice daily a diet consisting of corn silage, alfalfa hay, wet corn gluten feed, whole cottonseed, and grain mix. Treatments included daily abomasal infusion of 0 or 400 g/d casein. Cows received 320 g/d glucose through continuous abomasal infusion to make energy less limiting than protein for animal performance, thereby increasing responsiveness of the cows to the casein treatment. The experiment was conducted over 38 d, and it included 14 d for adaptation to facilities and diet and three 8-d periods. Milk, urine, and feces samples were collected from individual cows 3 times daily to evaluate milk production and composition and nitrogen retention. Abomasal casein infusion had no effect on milk yield and composition with the exception of milk protein percentage and milk urea nitrogen (MUN), which were increased significantly ($P < 0.01$) when casein was supplemented. Nitrogen retention ($P = 0.03$) and urinary N excretion ($P < 0.0001$) were increased and fecal N excretion ($P = 0.002$) was decreased by post-ruminal casein infusion. These results suggest that casein has potentially positive effect on stimulating protein deposition and can be used as an effective way to alter nitrogen utilization in lactating dairy cattle. The response of key variables to casein indicated that a 4-d adaptation period is appropriate for evaluating responses to casein supplementation. In addition, our data provide elements that can be used in power analysis to estimate appropriate replication in future

experiments. Overall, data from this pilot study can help design future experiments to evaluate amino acid utilization by dairy cattle.

Key Words: casein, nitrogen efficiency, milk composition

Introduction

Several studies have evaluated the use of amino acids other than methionine and lysine by lactating cows (Rulquin and Pisulewski, 2006). In general, milk protein composition and whole body protein metabolism can be manipulated by either the total dietary protein or energy intake (MacRae et al., 2000). The amount of dietary nitrogen (N) can affect the amount of N excreted by cattle; therefore, dietary protein intake has a considerable effect on the efficiency of utilization of N (Satter et al., 2002). Indeed, more precise feeding of protein and balancing of rations for providing an optimal balance of essential amino acids can improve the efficiency of utilization of N (NRC, 2001). It has been reported that increased post-ruminal supply of protein and/or amino acids can improve milk and protein yield, muscle growth and N retention by enhancing the efficiency of nitrogen utilization in cattle (Castillo et al., 2000; Reecy et al., 1996). The major bovine milk protein is casein (Phillips and Williams, 2011), which is synthesized in the mammary epithelial cells (Csapo and Salamon, 2009). Casein makes up approximately 80% of the total protein in ruminant milk (Wang et al., 2017), and it is easily digested in the intestine (Inglingstad et al., 2010).

Research has shown that protein supplementation improves α-amylase secretion in the small intestine in ruminants (Richards et al., 2003; Wang and Taniguchi, 1998). The infusion of casein into the abomasum could increase the absorption of glucose and starch disappearing in the ruminant small intestine. A number of studies showed that post-ruminal casein infusion could improve dry matter intake (DMI) (Choung and Chamberlain, 1992), milk production (Vanhatalo

et al., 2003 a,b; Huhtanen et al., 1997; Choung and Chamberlain, 1993), and milk protein content and protein yield (Vanhatalo et al., 2003 a,b; Huhtanen et al., 1997) in cows receiving a basal diet of grass silage.

Our objective was to develop a model useful for evaluating amino acid use by dairy cows. Also, our study was designed to evaluate effects of casein on milk production, whole body protein deposition, organic matter digestibility (OMD), and dry matter digestibility (DMD) in cattle.

Materials and Methods

All experimental procedures involving cattle were approved by the Institutional Animal Care and Use Committee at Kansas State University.

Two ruminally cannulated Holstein dairy cows (599 ± 17 kg initial BW) were used in a 38-d experiment, composed of 14 d for adaptation to facilities and diet and three 8-d periods, with samples collected daily. The experiment used a switch-back design. With this design, cows were provided one treatment, then switched to the other treatment, then switched back to the first treatment. The two treatments were provided in opposite sequences to the two cows. Cows were housed in tie-stalls with ad libitum access to water and fed twice daily (0500 and 1700 h) a diet consisting of corn silage, alfalfa hay, wet corn gluten feed, whole cottonseed, and grain mix (Table 1) at 27 kg DM/d. Cows were observed daily and the health status of each animal was evaluated and recorded. The basal diet was formulated to provide limiting amounts of protein to create an amino acid deficiency. The diet contained adequate ruminally degradable protein to assure that changes in nitrogen recycling, which could be affected by treatments, did not affect ruminal microbial growth. To prevent energy from being limiting without increasing microbial protein supply, additional energy was supplied to cows through continuous abomasal infusion of

320 g/d glucose. The glucose was provided to increase the energy:metabolizable protein ratio and thus to emphasize amino acid deficiencies and increase the responsiveness of the cows to protein supplementation.

Treatments were abomasal infusion of 1) no protein supplementation (control), and 2) 400 g/d casein. The basal abomasal infusate containing casein and glucose was prepared daily as follows. To prepare infusates for each 12-h infusion period, 200 g of sodium caseinate (as is weight) was initially solubilized in approximately 1600 g of water by blender. Because the casein solution contained air bubbles, the solution was allowed to sit for 8 h for air bubbles to escape. Then, 160 g glucose was added and mixed until dissolved. Water was then added to bring the final weight of abomasal infusate to 2,000 g for each 12-h infusion period. For the cow on the control treatment, glucose was simply dissolved in water.

Treatments were provided as nearly continuous infusions into the abomasum to preclude ruminal degradation. Treatments were infused into the abomasum through Tygon tubing (i.d. = 3.32 mm; Saint-Gobain North America, Valley Forge, PA) passed through the ruminal cannula, the reticulo-omasal orifice, and the omasum and held in the abomasum with a circular rubber flange (10-cm diameter) at one end. A peristaltic pump (Model CP-78002-10; Cole-Parmer Instrument Company, Vernon Hills, IL) was used to make the infusions. The infusion lines were disconnected from the pump while the cows went to the milking parlor and then were reconnected when cows returned to their stalls.

Data and Sample Collection and Analysis

Feed delivered and feed refusals were recorded daily to measure feed intake. Samples of the TMR, orts, and feed ingredients (corn silage, alfalfa hay, wet corn gluten feed, grain mix, and whole cottonseed) were collected daily and frozen (−20°C) for subsequent analysis. Samples

were mixed within period to obtain composite samples, dried in a 55°C forced-air oven for 72 h to determine partial DM, ground to pass through a 1-mm screen (Thomas-Wiley Laboratory Mill Model 4, Thomas Scientific USA, Swedesboro, NJ), and stored for subsequent analysis. Samples of feed, orts, and feces were analyzed for ash by combustion at 450°C for 8 h.

Cows were milked 3 times daily (0000, 1000, and 1700 h). Milk weights were recorded at each milking, and milk samples from individual cows were collected (25 mL) at each milking, preserved with 2-bromo-2-nitropropane-1,3 diol, and stored at 4°C for analysis of milk components. Milk samples were analyzed for fat, true protein, lactose (B-2000 Infrared Analyzer; Bentley Instruments Inc., Chaska, MN), milk urea-N (MUN spectrophotometer; Bentley Instruments Inc.), and somatic cells (SCC 500, Bentley Instruments Inc.) at the Heart of America DHIA, Manhattan, KS. Body weight was measured at the start and end of the experiment to compute BW change.

Fecal and urine samples were collected 3 times daily (0730, 1030, and 1330 h). Fecal samples (200 g) were collected either when cows spontaneously defecated or directly from the rectum, if necessary, and then were mixed to obtain a single composite for each day. Urine samples (20 mL, mixed to obtain a composite for each day) were collected either when cows spontaneously urinated or following stimulation of the vulva to induce urination. Samples of urine (60 mL) were diluted with 15 mL of 1.5 M H2SO4 and frozen (-20o C) for analysis of creatinine and nitrogen. Urinary creatinine concentrations were measured using HPLC as described by Brake (2010). Urinary creatinine excretion was estimated as body weight (kg) × 29 mg/kg of BW, and daily urine volume were estimated from urinary creatinine concentration (Valadares et al., 1999). Samples of the feed, ort, and feces were analyzed for indigestible acid detergent fiber (IADF; internal marker for determining diet digestibility and fecal output). An in

situ experiment was performed to measure IADF. The samples (feed, ort, and feces) were weighed (approximately 0.5 g) into F57 filter bags (Ankom, Fairport, NY), placed in mesh bags (n = 32 for each mash bag), and inserted into the rumen of 3 heifers for 192 h of incubation. After 192 h, the Ankom filter bags were removed from the rumen, rinsed under running cold water by hand, and allowed to dry at room temperature. Then, the dried bags were analyzed for ADF (Goering and Van Soest, 1970) using the batch procedure of Ankom Technology Corp. For calculating digestibilities, abomasal infusates were not included as part of the intake. Fecal output was calculated as IADF intake divided by fecal IADF concentration. Nitrogen concentrations of casein, feed, ort, wet feces, and urine samples were determined through combustion (Nitrogen Analyzer Model FP-2000, Leco Corporation, St. Joseph, MI). Milk N was calculated by dividing milk protein yield by 6.38. Nitrogen retention was determined as the difference between N intake (feed - refusals + casein infused) and N lost as feces, urine, and milk.

Statistical Analyses

Treatments were arranged in a switch-back design and included 0 and 400 g/d casein. Data were analyzed using the MIXED procedure of SAS System 9.3 for Windows (SAS Inst. Inc., Cary, NC). Data were analyzed with a repeated measures analysis with a model including fixed effects of treatment, and day within period, and the interaction between treatment and day. Cow was included as a random effect. Significance was declared at $P \leq 0.05$ and tendencies at $0.05 < P \leq 0.10$. Treatment means were calculated using the LSMEANS option. Because data demonstrated that adaptation required 4 d, data from d 5 through 8 were used to analyze the overall effects of treatment. These analyses were completed as described above, but with only the data from d 5 through 8 included.

Results and Discussion

Our work was conducted as a pilot study to provide information about casein utilization by dairy cattle and to develop a model for evaluating amino acid use by high-producing dairy cows. We observed interactions between treatment and day for milk urea ($P = 0.02$; Figure 13), urinary N excretion ($P = 0.06$; Figure 17), N retention ($P = 0.06$; Figure 19, and N efficiency ($P = 0.09$; Figure 20) for the data from d 1 through 8. Examination of the responses over time suggested that a 4-d adaptation period would be appropriate for evaluating responses to post-ruminal protein supplementation. This conclusion was supported by a lack of interaction between treatment and day for any of the responses when data from days 5 through 8 were analyzed ($P \geq 0.93$). Data also indicated that a 4-day adaptation period was appropriate to allow cows to reach a stable response for milk protein percentage and milk fat percentage. The use of short adaptation periods is not problematic because ruminants adapt rapidly to changes in the post-ruminal supply of nutrients where there is no need for ruminal adaptation to take place (Hofmann, 1989). This is in agreement with the findings by Whitelaw et al. (1986) that examined the effects of four levels of casein (0, 200, 400 or 600 g/d) in four Ayrshire cows with a short adaptation period (5 d) for each treatment. The results indicated that milk yield and composition responded rapidly to abomasal infusion of casein and the major changes milk yield and composition were observed within 24 h. These authors suggested that short adaptation periods, considering no change in basal diet and intake level among treatments and high digestibility of casein in abomasum, can be adequate.

DMI and Lactation Responses

Due to necessity of 4-d adaptation periods, as discussed above, the data presented in Tables 2 and 3 are from d 5 through 8. Total dry matter intake and milk production and composition are presented in Table 2.

Total dry matter intake was not affected by casein supplementation. A meta-analysis of Martineau et al. (2016) investigated effects of supplemental casein in dairy cows, and they observed that DMI tended to increase by 0.18 kg/d with casein supplementation across 48 studies in the literature. With our limited number of observations, we would not have been able to detect a difference of 0.18 kg/d.

No differences in milk production were observed in response to casein administration. Fat yield and percentage were numerically greater when cows received casein with the responses being potentially important, but these effects lacked significance due to large variation and lack of adequate sample size. In terms of model development, we conclude that substantial increases in the number of observations would be required for evaluation of effects on milk fat concentration and yield.

Hanigan et al. (2004) demonstrated that dairy cows in late lactation, which received abomasal infusions of four levels of casein (200, 400, or 600 g/d) for 10 days, did not show any significant differences in fat percentage, but fat yield increased (quadratic, $P = 0.06$) for cows receiving casein supplementation. A study by Cohick et al. (1986) investigated the effect of post-ruminal infusion of 395 g/d casein compared with water in four Holstein cows. Results of this study indicated that casein supplementation decreased milk fat percentage and increased milk yield.

When casein was infused, milk protein percentage and milk urea nitrogen (MUN) were greater ($P < 0.01$) than for the control. Milk protein yield was not statistically different ($P = 0.42$) from control when casein was infused, but infusion of casein improved milk protein yield by about 4%, indicating that casein supplementation may have stimulated protein synthesis (Broderick et al., 1970), although our experiment was not designed to detect differences of this size. There is considerable evidence that free amino acids in blood are important precursors for milk protein synthesis (Guinard and Rulquin, 1994). Uptake of amino acids by the mammary gland depends on the levels of amino acids in blood and the rate of amino acid transport across the membrane (Guinard and Rulquin, 1994). Supplementing deficient essential amino acids into the intestinal digesta can improve milk production (Guinard et al., 1994).

Furthermore, somatic cell count (SCC) was greater ($P = 0.02$) for cows receiving post-ruminal casein infusions, although this effect was largely driven by high SCC for one cow in one period when it received the casein treatment.

Hurtaud et al. (1993), using 4 ruminally and duodenally fistulated Holstein cows fed a diet consisting of 70% forage and 30% concentrate and receiving low or high amounts of ruminally infused propionate, demonstrated elevated milk and protein yields in response to duodenal infusion of sodium caseinate. This suggests that casein supplementation increased supplies of amino acids that were limiting for milk protein synthesis. Broderick et al. (1970) used cows averaging 31 kg/d milk to evaluate effects of abomasal infusions of 800 g/day of methionine-supplemented sodium caseinate. Casein supplementation significantly enhanced milk protein concentration and milk protein yield. These authors suggested that the increases in milk protein were due to provision of amino acids that limited milk protein synthesis. Griinari et al. (1997) observed increases in milk yield (11%) and milk protein yield (10%) following

abomasal casein infusion in rumen-fistulated Holstein cows (184 d postpartum). They also suggested that the increased milk protein yield could be a consequence of improved amino acid supply. Clark (1975) suggested that enhanced milk protein after treatment with casein might be associated with: 1) supplementation of amino acids that are limiting for milk protein synthesis, 2) improvement in the supply of glucogenic amino acids (Miettinen and Huhtanen, 1997), and 3) alteration in hormonal status, such as growth hormone, prolactin, and insulin (Spires et al., 1975). Consistent with our observations, other authors have reported that post-ruminal casein infusions in cows fed grass silage based diets increased MUN (Vanhatalo et al., 2003 a,b; Huhtanen et al., 1997). The increases in MUN might result from catabolism of amino acids, which can significantly affect blood urea-N (Rooke et al., 1987) and a positive correlation between blood urea-N and MUN (Aguilar et al., 2012).

Martineau et al. (2017) conducted a meta-analysis based on 23 experiments that post-ruminally supplemented casein to lactating dairy cows. They found that casein infusion increased milk true protein concentration and milk and component yields; however, supplemental casein decreased milk concentrations of fat and lactose. Results also illustrated a positive effect of casein on nitrogen retention and an increase in milk and blood urea concentrations. Elevations of urea suggest that some of the AA, following administration of casein, were catabolized, resulting in elevated urea synthesis.

Nitrogen Retention

Nitrogen retention was measured as an estimate of lean tissue deposition and was used in conjunction with milk protein yield to calculate the efficiency of N utilization. Table 3 shows the nitrogen retention responses of dairy cows to supplementation with casein. By design, the casein treatment increased ($P = 0.008$) total N intake. Abomasal infusion of casein increased N excreted

($P < 0.0001$) in urine by 29 g/d and reduced fecal N output by 14 g/d compared to control. Similarly, Bruckental et al. (1997) studied the effects of abomasal infusion of casein on nitrogen balance in Holstein steers, and they found that fecal N was significantly lower for casein group compared to the control group. As a potential explanation, casein may have increased small intestinal starch digestion, perhaps related to increased pancreatic alpha-amylase (Brake et al., 2014); therefore, microbial N production in the large intestine might have been decreased as a result of less starch being available as a fermentable substrate. There were no differences between the control and casein groups for OM or DM digestibility (Table 3).

In general agreement with our observations, Cohick et al. (1986), working with rumen-fistulated Holstein cows that received 0 or 395 g/d casein abomasally, showed total nitrogen intake (55 g/d), absorbed nitrogen (54 g/d), urinary nitrogen excretion (28 g/d), and milk nitrogen (13 g/d) were increased through abomasal casein infusion. These authors also indicated that casein tended to increase nitrogen retention. Elevated urinary N excretion might suggest that AA supplied by casein were in excess of the cow's requirement for protein synthesis (Blom et al., 2016) and therefore increased deamination and greater urinary losses of dietary protein were taking place (Vik-Mo et al., 1974). Huhtanen et al. (1997) also indicated a significant increase in urinary N excretion in lactating Ayrshire cows in response to abomasal casein infusion (Huhtanen et al., 1997).

Nitrogen retention was significantly augmented ($P = 0.03$) with casein supplementation compared with control. Consistent with our observations, other authors have reported that casein infusion into the abomasum increased N retention in steers (Titgemeyer and Merchen, 1990; Houseknecht et al., 1992; Bruckental et al., 1997).

Productive nitrogen and nitrogen efficiency were not affected by treatment, although the effect of casein supplementation on productive nitrogen was larger than the significant effect of casein on nitrogen retention. It is important to note that increases in N retention, instead of alterations in milk N and milk synthesis, in response to supplement casein in current experiment can imply that cow does not use the supplemental protein just for milk synthesis, suggesting that cows might tend to partition protein to optimize her productivity or it might reflect that the basal diet has already met the mammary gland's ability to respond to amino acid supply. Whitelaw et al. (1986) also illustrated progressive increases in milk N for 200, 400 or 600 g/d by 42%, 23%, and 15%, respectively, while the productive nitrogen efficiency (milk N plus retained N) was consistently increased across supplementation levels by 64%. Because supplemental protein can be used for other productive functions such as protein deposition instead of being used just for milk synthesis, it is important to consider N retention as part of the response by the lactating cow.

Conclusions

The results of this experiment demonstrate that post-ruminal casein supplementation is a useful model for evaluating efficiency of protein utilization in lactating dairy cows. Supplementation of casein led to increased urine N output, but increased productive N. Based on increases in nitrogen retention and milk protein percentage, casein supplementation improved whole body protein deposition, presumably by providing an increased supply of amino acids. The responses over time for key variables indicated that a 4-d adaptation period was appropriate. Data from this experiment can be used in power analyses during development of future experiments to ensure adequate replication is available to evaluate treatments.

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Table 5-1. Composition of experimental diet

Ingredient	% of DM
Corn silage	19.89
Alfalfa hay	21.85
Wet corn gluten feed	24.37
Whole cotton seed	5.01
Grain mix[1]	28.88
Nutrient composition	
Crude protein	14.7
OM	92.5

[1]Composition: 90.5% ground corn, 2.86% sodium bicarbonate, 4.29% calcium carbonate, 1.07% trace mineralized salt (> 95.5% NaCl, 0.24% Mn, 0.24% Fe, 0.05% Mg, 0.032% Cu, 0.032% Zn, 0.007% I, and 0.004% Co), 0.71% MgO, 0.039% Vitamin A premix (30,000 IU/g), 0.027% Vitamin D premix (30,000 IU/g), 0.29% Vitamin E premix (44 IU/g), 0.18% Zinpro 4-plex (5.15% zinc from zinc methionine, 2.86% manganese from manganese methionine, 1.80% copper from copper lysine, and 0.36% cobalt from cobalt glucoheptonate), and 0.036% Se premix (600 ppm Se).

Table 5-2. Effect of casein supplementation on dry matter intake and milk production and composition from day 5-8

	Treatment			*P*-value	
Item	Control	Casein	SEM	Treatment	Treatment × Day
Total DMI, kg/d	22.4	22.8	0.93	0.64	0.81
Dietary DMI, kg/d	22.1	22.1	0.93	1.00	0.81
Glucose DMI, kg/d	0.291	0.291	-	-	-
Casein DMI, kg/d	-	0.385	-	-	-
Milk yield, kg/d	31.5	32.0	0.97	0.67	0.93
Milk protein yield, kg/d	1.05	1.09	0.03	0.42	0.93
Milk protein, %	3.31	3.44	0.11	0.01	0.90
Milk fat yield, kg/d	1.13	1.24	0.10	0.45	0.83
Milk fat, %	3.63	3.92	0.42	0.63	0.85
Milk lactose yield, kg/d	1.54	1.55	0.07	0.94	0.96
Milk lactose, %	4.90	4.85	0.05	0.28	0.81
Milk SNF yield, kg/d	2.86	2.92	0.10	0.68	0.95
Milk SNF, %	9.11	9.15	0.07	0.68	0.69
MUN, mg/dL	7.82	8.89	0.25	0.002	0.26
Milk SCC, per µL	12.9	25.9	4.70	0.02	0.74

Table 5-3. Effect of casein supplementation on N intake, excretion, balance, and efficiency and diet digestibilities from day 5-8

	Treatment			*P*-value	
Item	Control	Casein	SEM	Treatment	Treatment × Day
Nitrogen, g/d					
Total nitrogen intake	548.3	601.5	20.8	0.008	0.82
Dietary nitrogen intake	548.3	543.6	20.8	0.78	0.82
Casein nitrogen infused	0.0	57.9	-	-	-
Milk nitrogen	164.5	170.4	5.1	0.42	0.93
Fecal nitrogen	169.3	155.7	2.5	0.002	0.79
Urinary nitrogen	167.0	195.7	4.8	<0.0001	0.84
Nitrogen retention	48.4	78.9	13.3	0.03	0.83
Productive nitrogen[1]	214.1	247.2	16.6	0.17	0.66
Nitrogen efficiency[2], %	38.7	41.3	1.7	0.28	0.76
Digestibility, %					
DM	63.1	63.1	2.6	0.96	-
OM	64.7	65.4	2.6	0.55	-

[1] Productive nitrogen = Milk nitrogen + Nitrogen retention
[2] Nitrogen efficiency = Productive nitrogen:total nitrogen intake

Figure 5.1. (1-13) Effect of casein supplementation on dry matter intake, milk production and composition from day 1-8

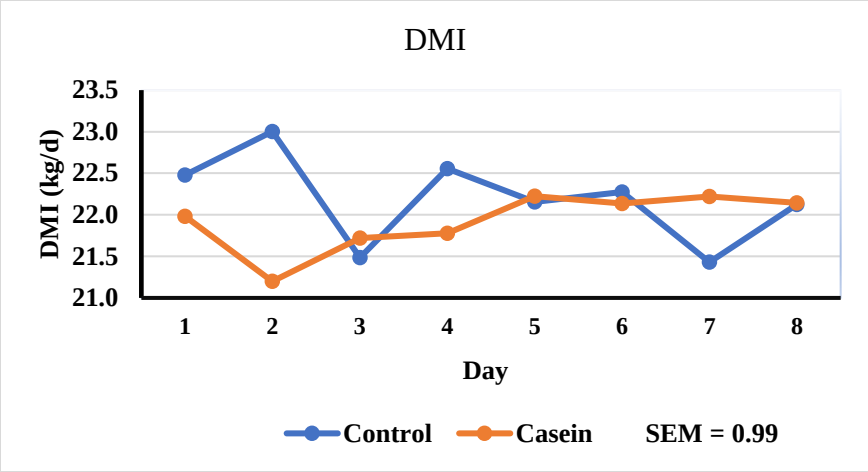

Figure 1. Effect of casein, *P* = 0.53; treatment × day, *P* = 0.33

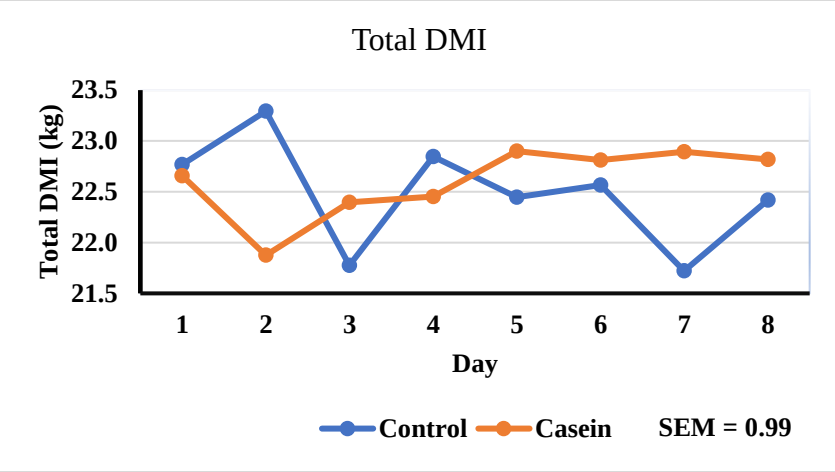

Figure 2. Effect of casein, *P* = 0.77; treatment × day, *P* = 0.33

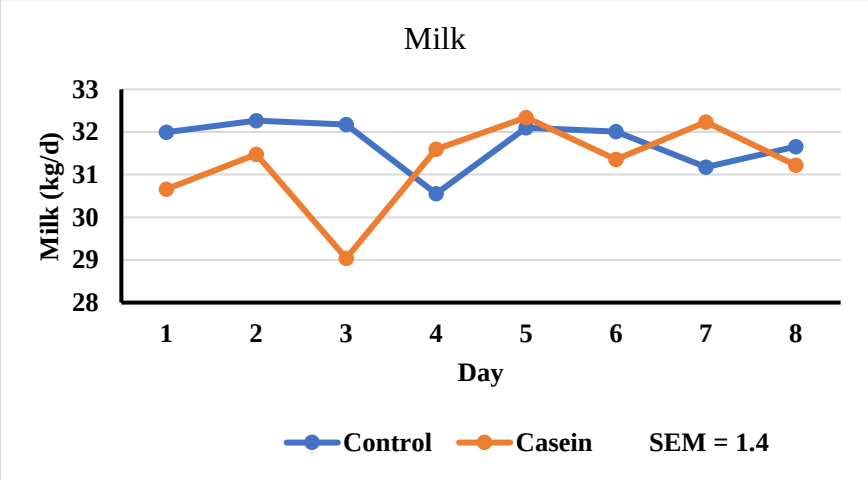

Figure 3. Effect of casein, *P* = 0.32; treatment × day, *P* = 0.73

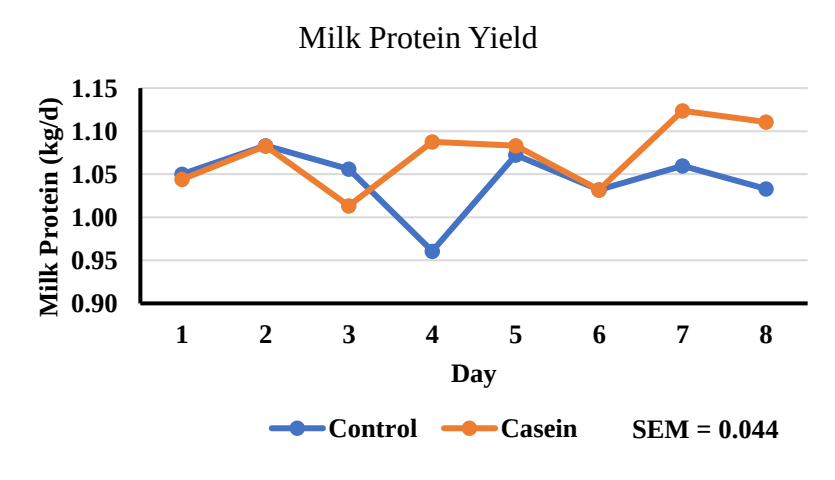

Figure 4. Effect of casein, *P* = 0.08; treatment × day, *P* = 0.75

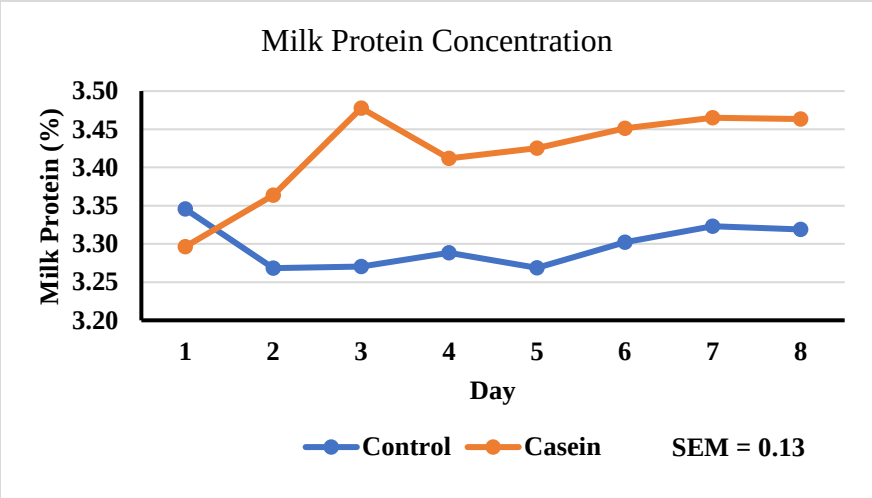

Figure 5. Effect of casein, $P < 0.0001$; treatment × day, $P = 0.32$

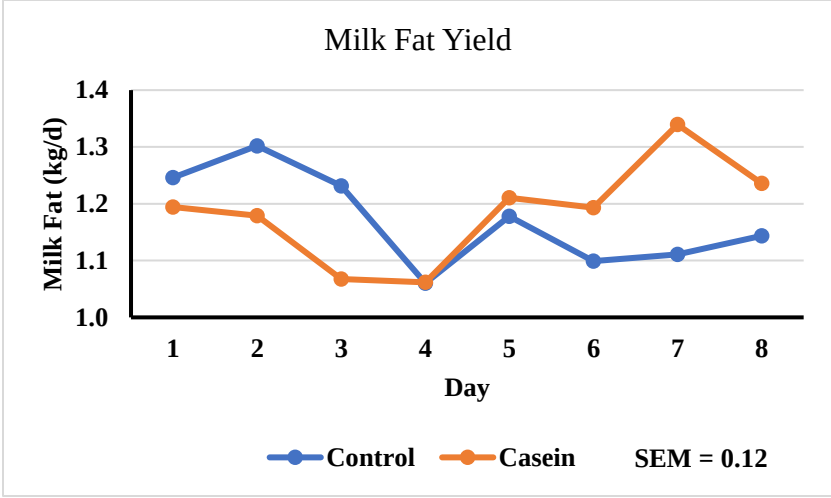

Figure 6. Effect of casein, $P = 0.90$; treatment × day, $P = 0.71$

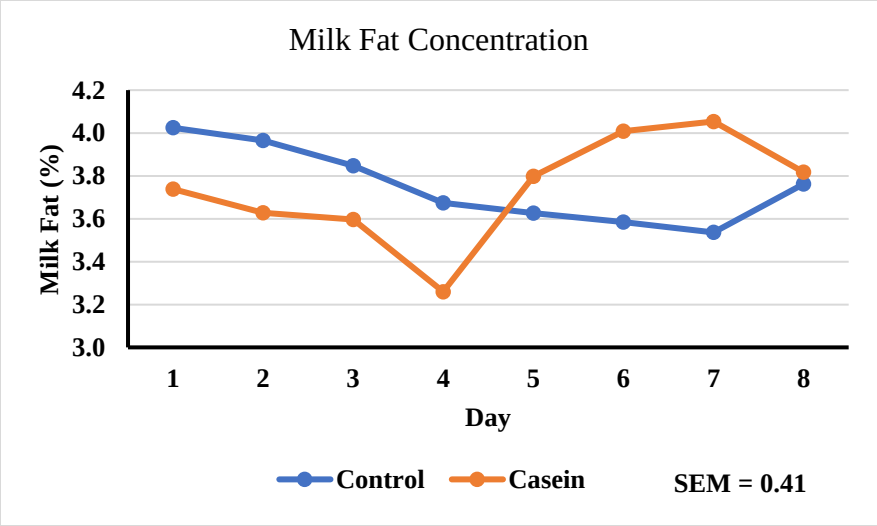

Figure 7. Effect of casein, $P = 0.97$; treatment × day, $P = 0.92$

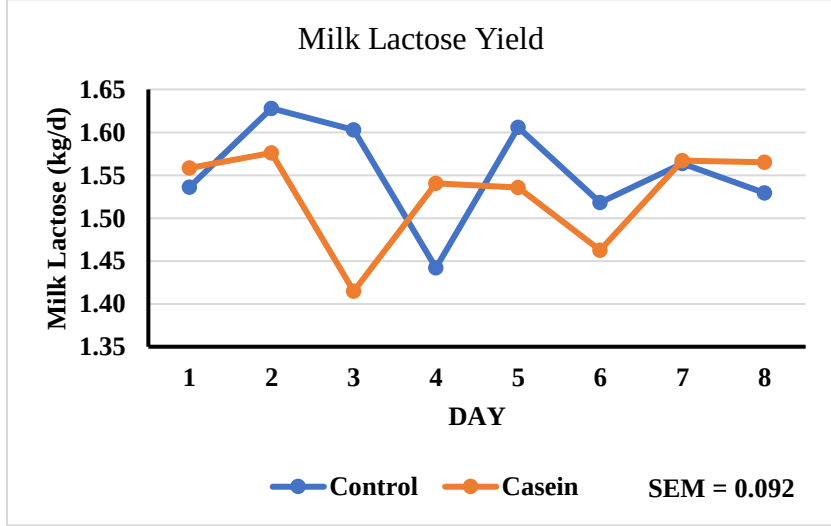

Figure 8. Effect of casein, $P = 0.49$; treatment × day, $P = 0.76$

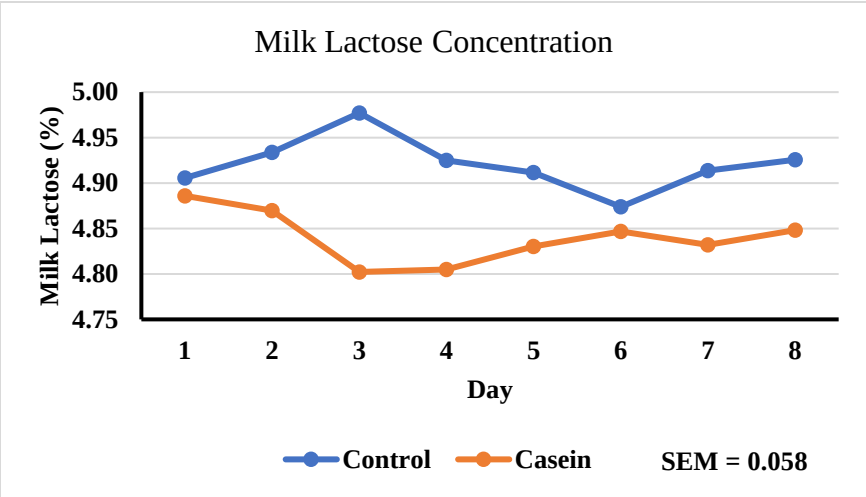

Figure 9. Effect of casein, *P* <0.0001; treatment × day, *P* = 0.37

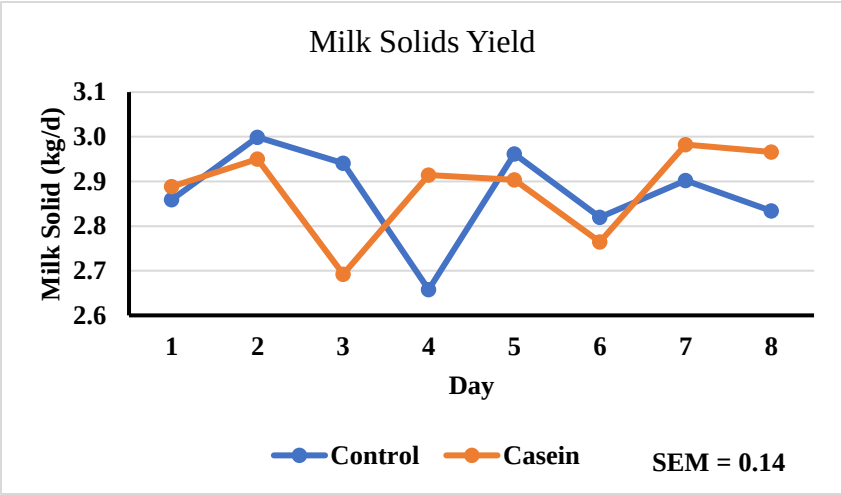

Figure 10. Effect of casein, *P* = 0.86; treatment × day, *P* = 0.78

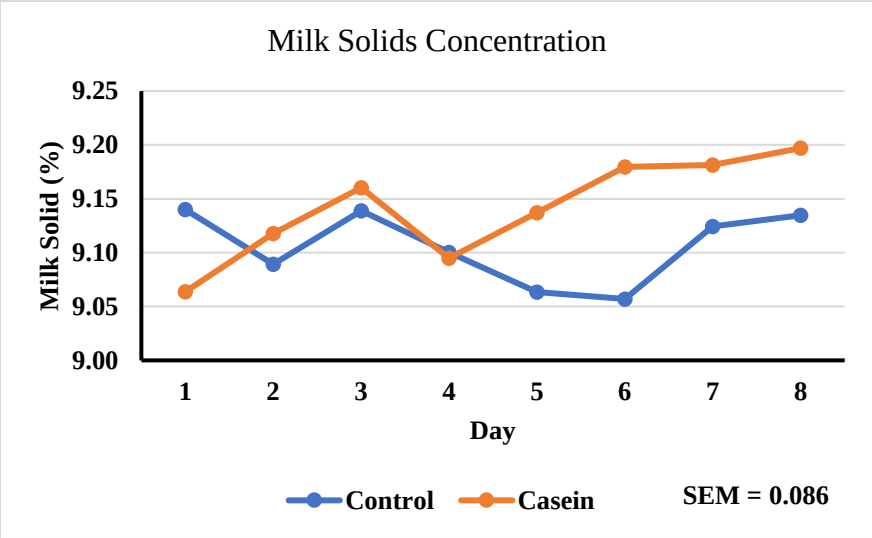

Figure 11. Effect of casein, *P* <0.0001; treatment × day, *P* = 0.89

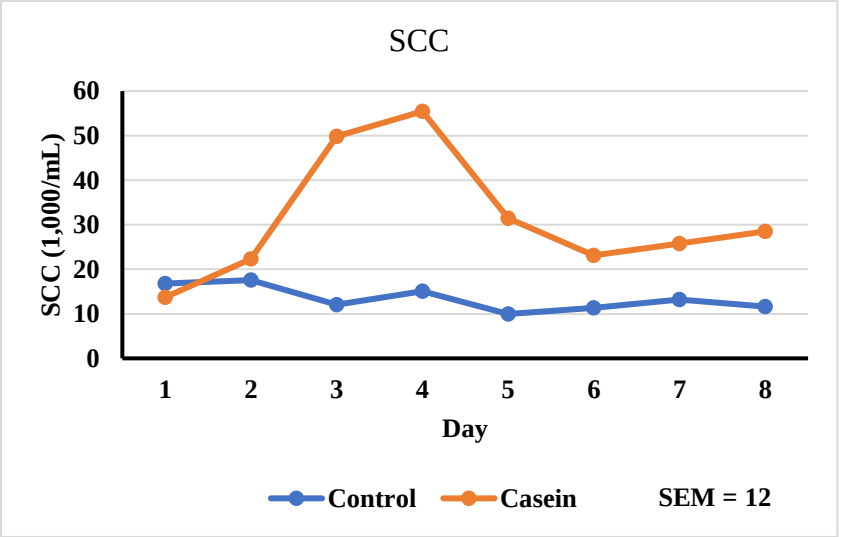

Figure 12. Effect of casein, *P* = 0.02; treatment × day, *P* = 0.24

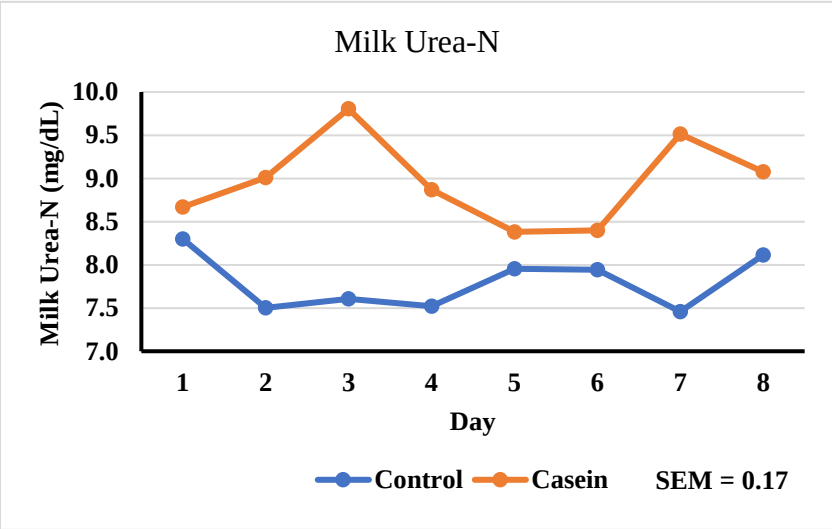

Figure 13. Effect of casein, *P* <0.0001; treatment × day, *P* = 0.61

Figure 5.2. (14-21). Effect of casein supplementation on N balance from day 1-8

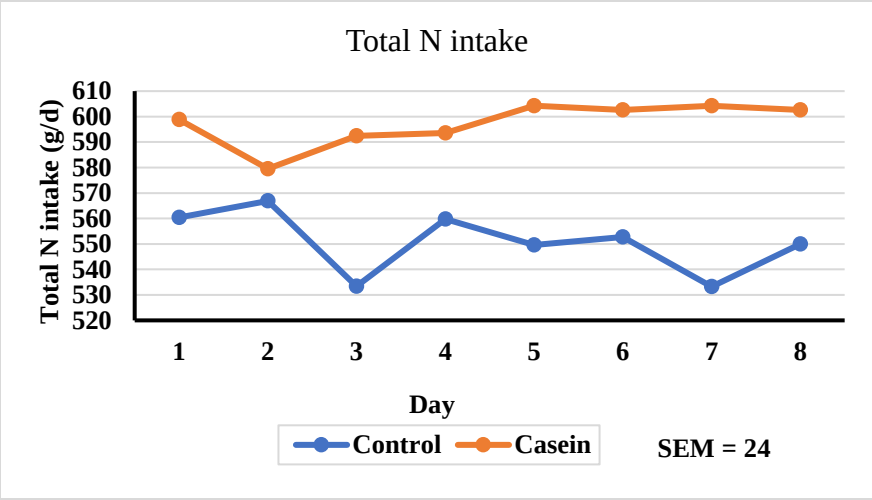

Figure 14. Effect of casein, $P < 0.0001$; treatment × day, $P = 0.33$

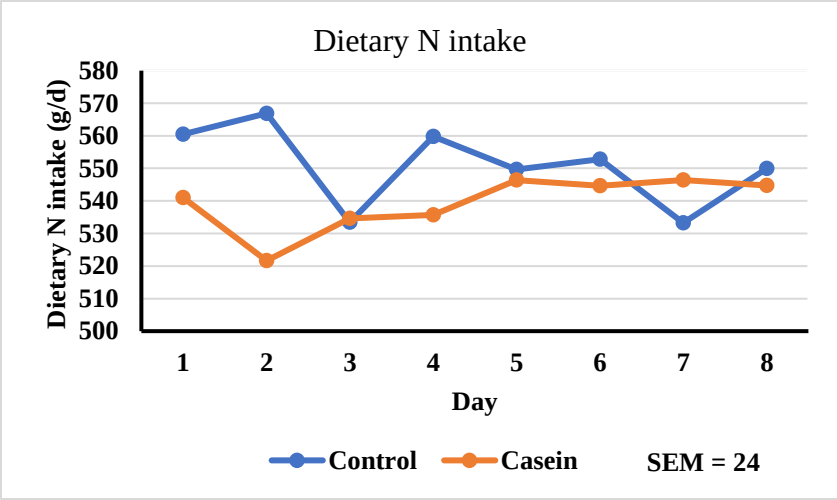

Figure 15. Effect of casein, $P = 0.18$; treatment × day, $P = 0.33$

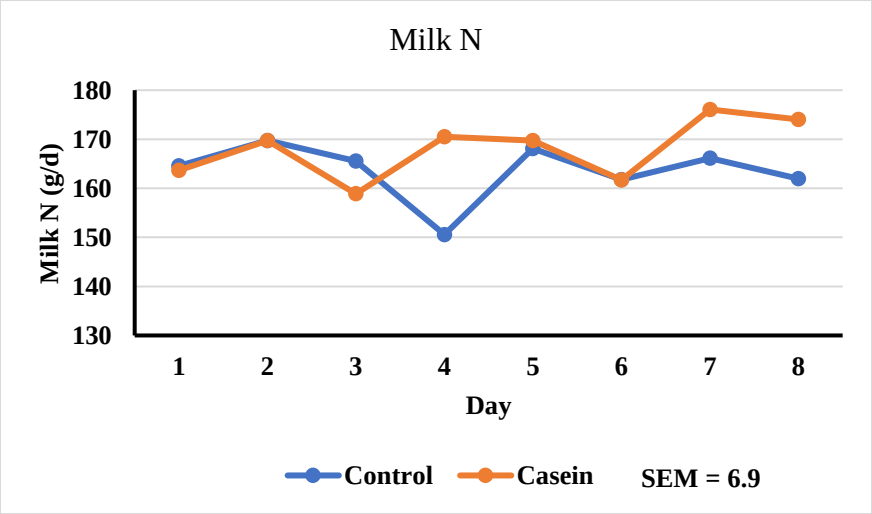

Figure 16. Effect of casein, $P = 0.08$; treatment × day, $P = 0.75$

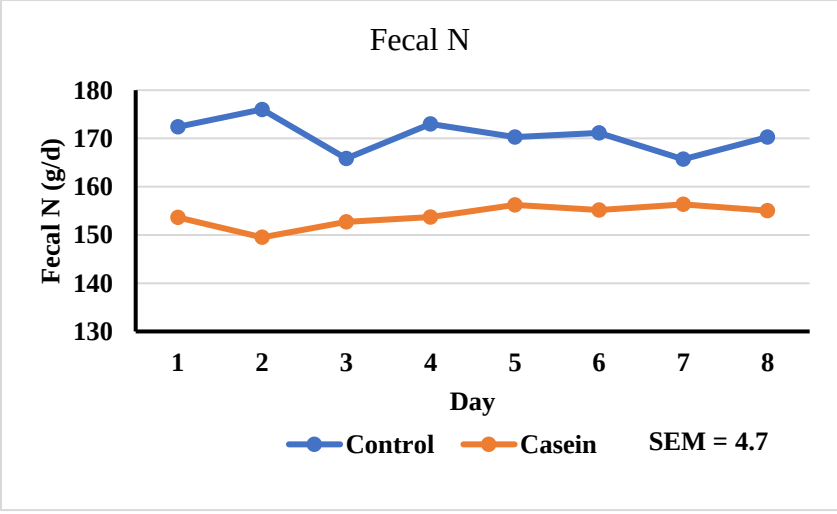

Figure 17. Effect of casein, $P = 0.01$; treatment × day, $P = 0.31$

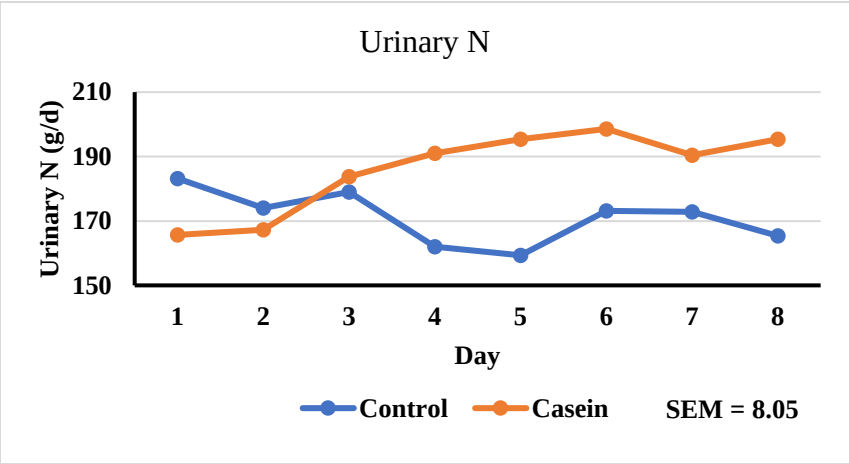

Figure 18. Effect of casein, $P = 0.0003$; treatment × day, $P = 0.06$

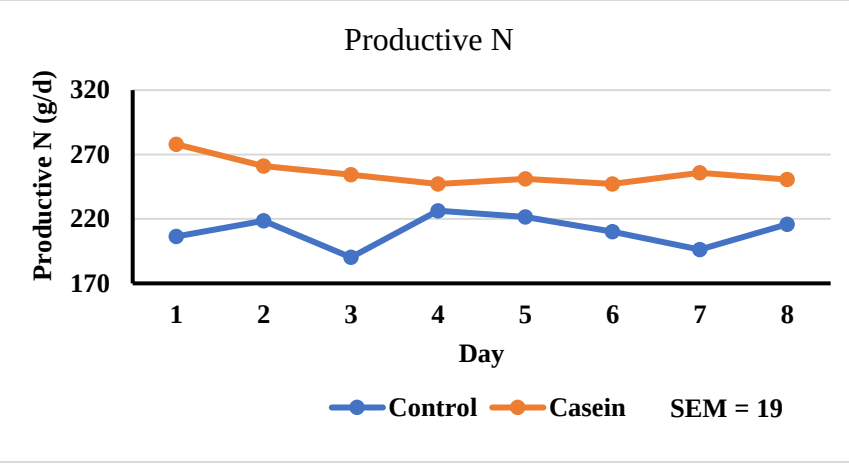

Figure 19. Effect of casein, $P = 0.0004$; treatment × day, $P = 0.21$

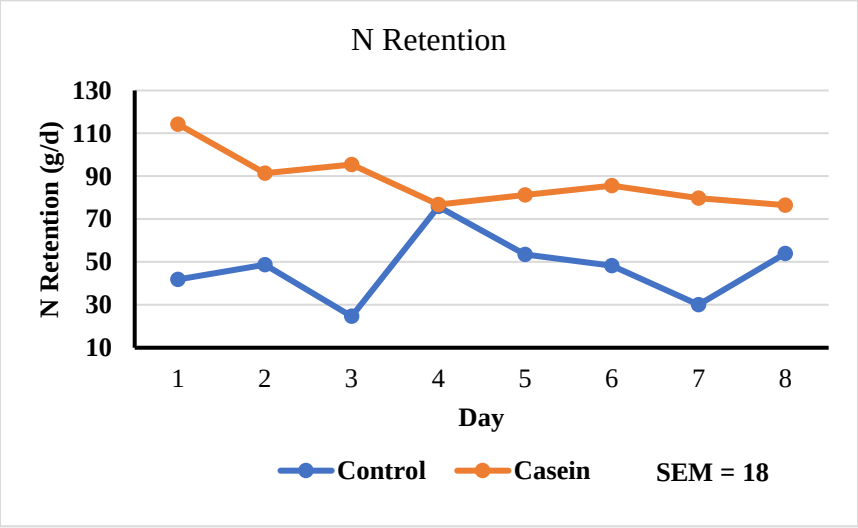

Figure 20. Effect of casein, $P < 0.0001$; treatment × day, $P = 0.06$

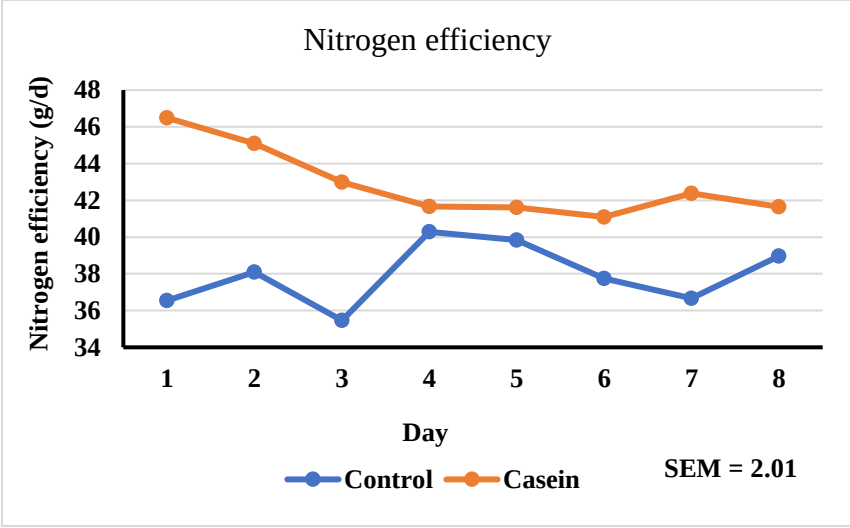

Figure 21. Effect of casein, $P = 0.001$; treatment × day, $P = 0.09$

Chapter 6 - Research Implications

GAA utilization in cattle

Creatine serves as an energy-storing molecule in muscle, and, although it is synthesized in the body from guanidinoacetic acid (GAA), the production rate of GAA, and consequently that of creatine, may not be adequate to maximize performance. The long term goals of the research were to improve the efficiency of the dairy and beef cattle industries by developing new information about methyl group utilization and creatine synthesis by cattle.

Two projects were undertaken to obtain some preliminary information as to how cattle respond to GAA supplementation with and without methionine. Because there was no data available about GAA utilization by cattle, this work was novel. The experiments determined the level of GAA most useful for providing creatine as well as the amount that might be useful for generating a model to evaluate methyl group deficiency.

We observed that large doses of GAA were not toxic and that GAA did serve as a precursor for creatine synthesis in cattle. We observed increases in plasma homocysteine (a marker of methyl group deficiency) when GAA was supplemented to cattle, but the increases were well below levels associated with clinical homocysteinemia, and the increase in plasma homocysteine could be eliminated by methionine supplementation, presumably because the methionine served as a source of methyl groups. The elevation in plasma homocysteine in response to GAA supplementation, along with an amelioration of this effect when methionine was supplemented, suggests that a useful model might be developed to assess efficacy of methyl group donors for cattle.

We evaluated the ability of methyl group donors or consumers to influence methionine-linked methylation reactions and consequently the efficiency of methionine use for protein

deposition. In the presence of supplemental methionine, supplemental GAA improved nitrogen retention (a measure of lean tissue growth), but this improvement was not observed when methionine was not supplemented. Thus, an increased creatine supply, under conditions where methyl groups are limiting, may not be likely to improve cattle growth.

Our results showed that total methylation reactions were not affected by GAA provision. Theoretically, GAA supplementation would increase methyl group consumption; thus, the lack of change in total methylation reactions by GAA supplementation in our experiment provided evidence that methylation reactions other than creatine synthesis, such as choline synthesis, must have been correspondingly decreased.

In our experiments, nitrogen retention was used as a measure for evaluating of protein deposition. Nitrogen retention was not affected by GAA supplementation when Met was not provided, but GAA linearly improved N retention in the presence of Met. GAA supplementation increased linearly plasma creatine concentration, suggesting that GAA was converted to creatine in cattle. Taken as a whole, these results support the concept that when methionine was deficient, supplemental GAA had no positive effects on protein deposition (lean tissue deposition). Thus, an increased creatine supply, under conditions where methyl groups are limiting, may not be likely to improve cattle growth. Our research has direct implications for both the dairy and beef cattle industries, because GAA may be an economical means of improving creatine status of cattle, which may improve performance of these animals.

The very low amounts of GAA in urine demonstrated that most of the supplemental GAA was used for creatine synthesis. The increases in urinary creatine concentrations in response to GAA supplementation provided insights into linkages between increased creatine synthesis and plasma creatine concentration. When the body is faced with increased GAA supply, much of the

GAA is methylated to creatine, but the excess creatine will be eliminated through renal excretion as a result of reduction in renal reabsorption of creatine. Further research is necessary to fully elucidate the relationship between plasma concentration and renal reabsorption of creatine and GAA following GAA provision to cattle.

In response to GAA supplementation, the increase in plasma concentrations of arginine, which is a precursor used for GAA/creatine synthesis, showed that GAA can have a sparing effect on arginine. Therefore, GAA would provide beneficial effects to growing cattle by serving as a precursor to creatine or by sparing arginine, an amino acid that is the precursor to GAA. If creatine synthesis limits muscle deposition, then GAA supplementation might be a means of improving beef production when methyl group sources such as Met are supplemented. Methyl group sources other than Met may also be useful to ensure that GAA supplementation does not create a methyl group deficiency, although we did not evaluate any methyl group sources besides Met.

Methyl group utilization is currently an important topic in dairy nutrition, and our research is an initial step toward development of a model to compare the efficacy of different methyl group sources in cattle. The results of our experiment can open a new window of opportunity to better understanding of methyl group utilization to match nutrient supplies with animal requirements, particularly methionine (a frequently limiting amino acid) and also to improving the efficiency of the dairy and beef cattle industries. This research provides a promising opportunity for improving the efficiency of beef production by developing new information about GAA and creatine metabolism to increase growth performance. It is also of interest that, due to the positive effect of GAA on nitrogen retention, administration of GAA plus methionine may increase growth rate, which could reduce the length of the growing phase in

beef calves, which could increase the profitability and income for producers. Indeed, it is important to understand whether GAA supplementation can improve the growth in calves by sparing amino acids. Therefore, future research should investigate to explore the relationship between GAA supplementation and growth of beef calves.

In ruminant animals, there is no work that has assessed the efficiency of GAA use by lactating dairy cows or transition cows. Creatine supplementation can prevent fat accumulation in liver when rats are fed a high-fat diet. Fatty liver (hepatic lipidosis) is a common condition in dairy cow during the transition period and hepatic export of fat is deemed the key factor in hepatic lipidosis, therefore, the utilization of GAA can be used as a new tool to assess the efficiency of GAA supplementation on fat accumulation in liver in transition dairy cattle to improve herd health, performance and decrease economic losses due to disease. In addition, our studies indicated that GAA was able to increase creatine production, therefore, our results will help establish the model to consider the effect of GAA on placental creatine metabolism, uterine tissues (where energy demand is increased during pregnancy), and fetal growth to improve pregnancy outcomes.

This is an area of research that needs further exploration to provide useful insights of GAA and creatine metabolism and their effects in ruminants.

Comparison of two different sources of rumen protected methionine

Methionine is often considered one of the most limiting amino acids for optimized dairy cow production. Feeding free methionine is typically not considered an acceptable practice for ruminants due to the high rate of degradation within the rumen; methionine is therefore protected from rumen degradation to provide greater amounts to the site of absorption. Physical protection minimizes the access of the methionine to the rumen environment and results in reduced

degradation for well protected products. There are two methods, physical and chemical protection, to protect the methionine from rumen degradation to provide higher concentrations at the site of absorption. In Chapter 3, key response criteria were milk protein percentage and yield and plasma methionine concentrations to evaluate the relative effectiveness of feeding different levels of two different protected methionine sources. Our experiment showed that supplementation of Met improved milk protein percentage and yield without differences between Smartamine and NTP-1401; therefore, these two sources were generally similar in their ability to provide metabolizable Met to the cows. Although Met availability did not differ between the two methionine sources, Smartamine increased plasma D-Met, whereas NTP-1401 increased plasma HMTBa. Therefore, it is important, particularly for declaration of true methionine amount in feed, to consider that plasma Met concentration may not be a good criterion for estimating the true methionine availability.

Casein utilization by dairy cows

Casein is the predominant protein in milk; therefore, casein supplementation can improve protein synthesis and nitrogen efficiency by increasing amino acid supply. In Chapter 4, a pilot study was designed to develop a model useful for evaluating protein use by dairy cattle and to evaluate responses in milk protein output and whole body protein deposition, especially over short periods, to gain preliminary information about experimental variation that can be used to design future experiments. The data demonstrated that milk protein percentage and N retention were greater when casein was infused, suggesting casein stimulated protein synthesis by supplying amino acids. Also, based on increases in N retention with no alteration in milk N secretion, it was demonstrated that dairy cattle do not use supplemental protein only for milk synthesis. This study also showed that the use of short periods is appropriate because the cows

adapted relatively quickly to changes in the amount of casein supplemented post-ruminally. Finally, estimates of variation are available for power analyses to ensure adequate replication for future experiments.

Appendix A - **Effect of Met and GAA supplementation on plasma homocysteine and cysteine (d 6, 8, and 10)**

	0 Met			6 g/d Met								
	GAA, g/d							*P*-value[1]				
Amino acid, µ*M*	0	7.5	15	0	7.5	15	SEM	Met	G-L	G-Q	Met × G-L	Met × G-Q
Homocysteine[1]												
Day 6	12.4^{a}	13.1^{a}	13.4^{a}	9.5^{b}	10.0^{b}	9.5^{b}	1.2					
Day 8	12.1^{a}	11.9^{ab}	12.1^{a}	8.9^{c}	10.1^{abc}	9.8^{bc}						
Day 10	11.7^{bc}	13.7^{a}	12.7^{ab}	9.9^{c}	9.9^{c}	10.0^{c}						
Over all days	12.1	12.9	12.7	9.4	10.0	9.7	1.1	<0.01	0.19	0.17	0.70	0.89
Cysteine[2]												
Day 6	94.4^{a}	96.1^{a}	95.5^{a}	111.2^{b}	110.6^{b}	107.9^{b}	5.1					
Day 8	94.3^{a}	99.0^{ab}	95.0^{a}	106.8^{bc}	113.7^{c}	113.0^{c}						
Day 10	92.4^{a}	98.2^{a}	95.6^{a}	110.5^{b}	113.9^{b}	110.8^{b}						
Over all days	93.7	97.8	95.4	109.5	112.8	110.6	4.8	<0.01	0.54	0.12	0.88	0.89

[1] Day, *P* = 0.02; Day × Met, *P* = 0.15; Day × GAA, *P* = 0.84; Day × Met × GAA, *P* = 0.02.

[2] Day, *P* = 0.65; Day × Met, *P* = 0.78; Day × GAA, *P* = 0.30; Day × Met × GAA, *P* = 0.30.

[a,b,c] Means within day not bearing a common superscript differ (P < 0.05; using Slice option

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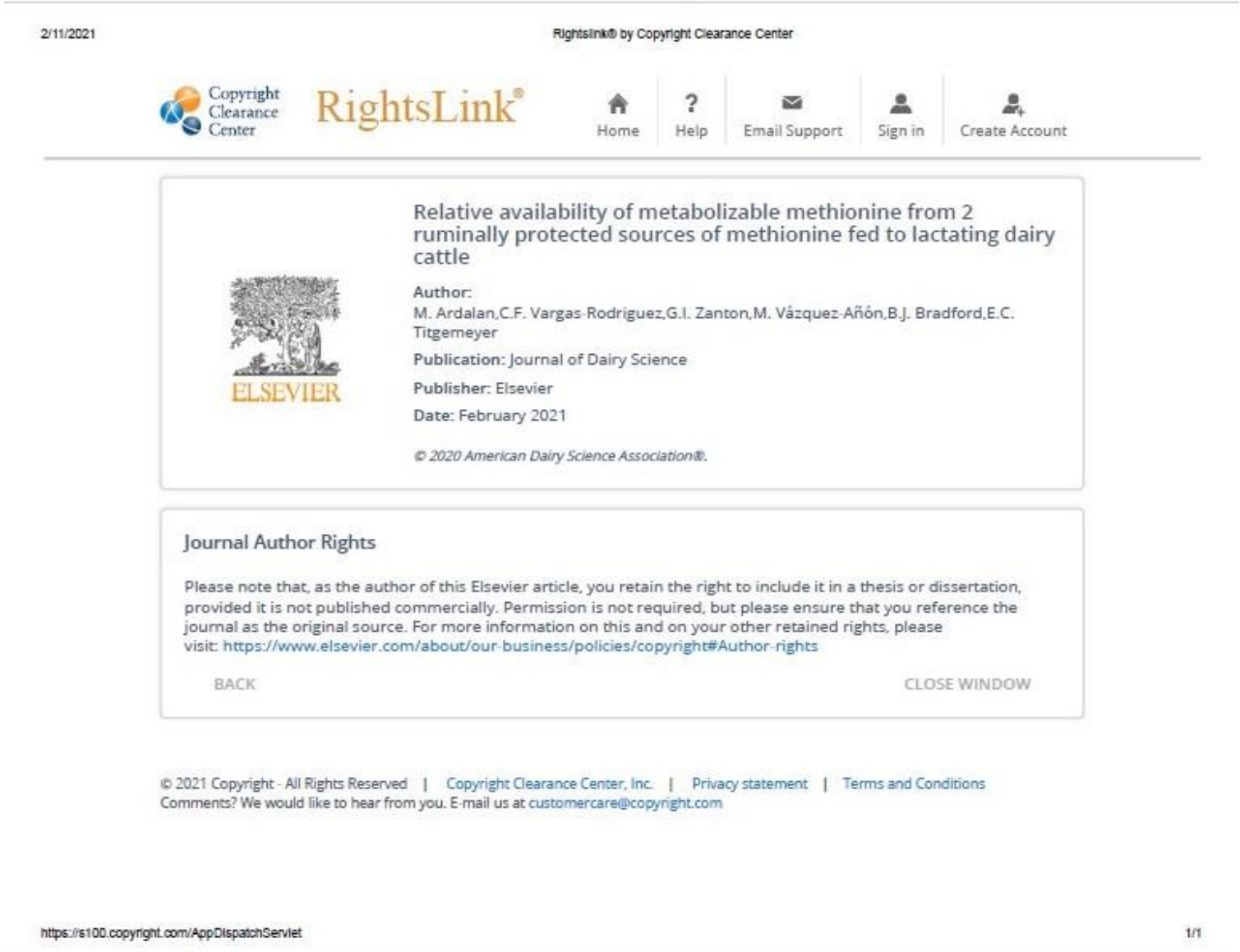

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Relative availability of metabolizable methionine from 2 ruminally protected sources of methionine fed to lactating dairy cattle

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